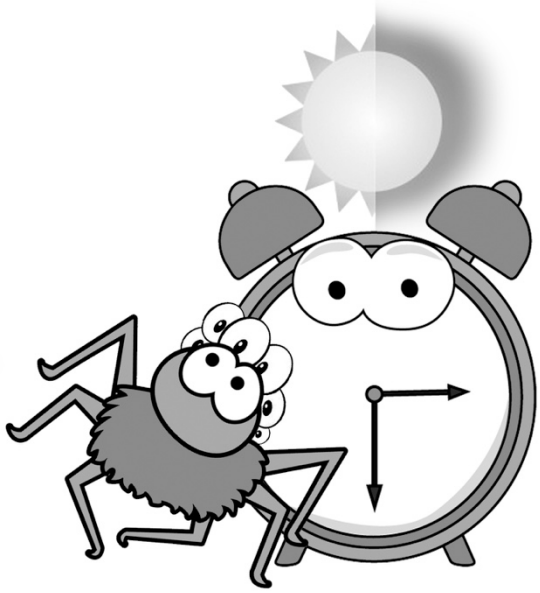
TEMPORAL BEHAVIOUR IN JUMPING SPIDERS

(ARANEAE, SALTICIDAE)



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Abstract

A circadian rhythm of daily activity and an annual rhythm are two primary rhythms recognized among animals, where an external signal (zeitgeber), typically light/dark daily rhythms or seasonal cycles acts to entrain the rhythm of an internal oscillator. In order to synchronize with daily factors, the circadian system should contain a photoreceptor (s) which may be placed either in the visual (ocular pathway) or in non-visual (extraocular pathway) organs. In addition, non-photic factors (like thirst), may interact with the circadian cycle in determining the operation of biological clocks. Generally, a circadian rhythm should persist in the absence of a periodic zeitgeber cue, but some behavioural processes in arthropods do not seem to follow this idea of a classic internal oscillator. In these cases, the timing mechanisms are referred to as 'hourglass' models. In nocturnal animals, light inhibits activity, whereas it boosts activity in diurnal animals. Direct effects of light result in a phenomenon called "masking effects" which veil the actual entrained circadian activity rhythm (s). With more than 6000 described species in more than 600 genera, the Salticidae is a large family of diurnally-active cursorial spiders. I investigated aspects of the temporal behaviour of salticids in this thesis in a series of experiments measuring activity patterns over a period of several days. I begin with a series of comparative experiments using four salticid species to investigate the effects of temperature and light duration on the locomotory activity patterns, depending on sex and age. I found that circadian activity patterns differed widely between species and, within species, also differed depending on both temperature and photoperiod. I then explored the effect of reproductive status and food-related effects (e.g., hunger, thirst) on the locomotor activity of female *Portia fimbriata* and *Marpissa marina*. Thirsty spiders, and those fed preferred prey prior to testing had increased activity levels, while females that had recently laid eggs became almost inactive. I then investigated short-term temporal effects on salticid behaviour across different light regimes simulating different latitudes. I found no support that *M. marina* adhere to hourglass models of timekeeping, instead relying on circadian clocks with a period of close to 24 h to maintain a thoroughly resilient diurnal pattern of behaviour despite showing differential responses to phase advances and phase delays. Subsequently, I described the timing of locomotor activity at different light levels within a 12:12 LD simulation in *Marpissa marina*, finding that anticipation of the start

and end of daylight hours is related to light intensity during daylight hours. Additionally, low level moonlight illumination significantly increased activity compared to total darkness. A clear masking effect on the spiders' clock was found under bright light, in which condition *M. marina* were no longer able to anticipate lights-on and lights-off. I then investigated Aschoff's rule using *M. marina* in conditions of constant darkness and constant light at different intensities to determine the effect of different light intensities in their free-running period. Depending on the intensity of light, constant light can disrupt circadian rhythmicity in *M. marina*, with a variety of responses exhibited, ranging from reduced locomotion and an increase in tau (the difference angle between the onset of activity and light-on time) to complete arrhythmicity (loss of daily rhythms over successive cycles). Finally, in a series of eye covering experiments, I investigated which of the four pairs of eyes were capable of entrainment, finding that the 'secondary' eyes, but not the 'primary' eyes were used to synchronize the circadian clock.

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Preface

The data chapters of this thesis were written as a collection of stand-alone papers for submission in peer-reviewed journals and Chapters 2 and 7 have already been submitted. In all other chapters, I developed the experimental design, carried out the experiments, analysis and writing. All submitted chapters are unaltered except to fit thesis style.

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Chapter 2: Tork, P. 2018. The effect of photoperiod and temperature on the locomotory activity of four species of jumping spiders (Araneae, Salticidae). *Journal of Arachnology.*

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Chapter 7: Tork, P. 2018. Pathways of ocular entrainment in *Marpissa marina* (Araneae, Salticidae). *New Zealand Journal of Zoology.*

"Science never solves a problem without creating ten more"

George Bernard Shaw

Chapter 1

Introduction

Two main rhythms in the behaviour of animals have been recognized: a rhythm of daily activity and an annual rhythm (Bradshaw & Holzapfel 2010), which are based on molecular 'clocks entrained (reset) by an external signal, or zeitgeber (Rensing et al. 2001). The daily (circadian) rhythm enables animals to follow daily changes in their environment, while a photoperiodic timer which is particularly acute at higher latitudes enables animals to anticipate seasonal changes (Bradshaw & Holzapfel 2010). Photoperiodism acts as a go/no-go seasonal switch that underlies migration patterns, dormancy, development, and reproduction, while circadian rhythms can be entrained to daily dawn and dusk (Pittendrigh 1960, 1965, 1981b; Aschoff 1965; Nijhout 1994; Bradshaw & Holzapfel 2007a). In order to synchronize with daily dawn and dusk, the circadian system should contain a photoreceptor (s), which may be occur either in the visual (ocular pathway) or in non-visual (extraocular pathway) organs. As an anticipatory key to time-related events in animal's life history, photoperiodism relates to how animals assess the length of the day, which is affected by latitudinal and seasonal changes (Bradshaw & Holzapfel 2007a). For example, long summer days at higher latitudes may affect the onset of activity in animals whose behaviours are under circadian clock control (Pittendrigh 1993; Bradshaw & Holzapfel 2007b) although the same species in different latitudes may exhibit different reactions to these seasonal changes.

The role of entrainment is to certify that the rhythm keeps a fixed phase relationship with the environmental cycle and agitates rhythmic outputs at the proper daily time (Hardin 2005). Determining endogenous periodicity requires controlling for all exogenous cues. These experiments are usually performed in constant conditions and measure locomotor activity in response to photic and other ambient changes (Page & Larimer 1972; Daan & Aschoff 1975; Rieger et al. 2003). Aschoff's (1981) 'rule' states that; nocturnal animals keep their endogenous (circadian) periodicity rhythm under DD (constant darkness), albeit this may deviate somewhat from a 24 h cycle, while in LL (constant light) they show arrhythmic behaviour. He demonstrated that in many species, the circadian period depends on the light intensity, such that an increase

in light intensity will lead to a shortening of the period in many diurnal animals, and a lengthening of the period in nocturnal species (Aschoff 1981).

Different animals may response differently to changes in daylight hours due to different clock mechanisms regulating temporal behaviour. For example, some behavioural and physiological processes in arthropods (e.g., diapause or reproductive phase in aphids in response to seasonal changes; Lees 1966, 1973) do not seem to follow a classic internal oscillator (Saunders et al. 2002). In these cases, the timing mechanisms are referred to as 'hourglass' models. In these models, the periodic cycle 'dies out' within a cycle in the absence of an external input (e.g., dawn), whereas endogenous circadian clocks typically require a number of days to reach a complete phase shift (Daan 1987; Bradshaw et al. 2003). If the phasing of a behaviour is under endogenous circadian clock control, under constant conditions the behavioural rhythm should continue (free-run) with the period of the internal clock. On the other hand, if a behaviour is controlled by exogenous factors, that behaviour should happen instantly (or shortly) after dusk or dawn, and the rhythm cannot persist under constant conditions. Therefore, an external signal, such as light, can be used to distinguish between circadian clock and hourglass models. If the behaviour is based on an hourglass mechanism, an advance or delay in light regime should produce a quick phase shift, whereas if timing relies on a self-sustaining oscillator, the onset of behaviour should occur with a delay relative to the new light regime (Biebach et al. 1991).

While the light/dark cycle is the primary influence to biological rhythms through entrainment, non-photic factors can interact with the circadian cycle in determining the operation of biological clocks. In addition to light, temperature and restricted food may influence the photoperiodic timer (Saunders 1966a, b), and may be crucial among species that live in the tropics, where daylight changes are minimal over the year, so photoperiod may be a less informative predictor of impending climate than in temperate areas.

How light-sensitive biological clocks are has received relatively little attention in the literature. It has been demonstrated that continuous dim light strongly influences the period and the power of the free-running rhythm, and continuous light exceeding a certain threshold may provoke arrhythmicity (Aschoff 1979; Konopka et al. 1989). Yet, the direct effects of light on physiology as opposed to the circadian clock (s) and activity can be hard to distinguish because light can result in 'masking', or veil, the actual entrained circadian activity rhythm (s) (reviewed in Mrosovsky 1999) and total

darkness and intense light have a masking effect on the activity of some animals. On the other hand, in some organisms, moonlight influences nocturnal activity, as well as diurnal activity (Fernandez-Duque & Erkert 2006) because of the extreme light sensitivity of their circadian clock(s). At the appropriate intensity of light, animals can anticipate and respond to regular events (e.g., in rabbits, Jilge 1995, and in *Drosophila*, Wheeler et al. 1993), while strong illumination or total darkness may lead to a breakdown of clock function, resulting in arhythmicity or a masking of activity (Helfrich-Förster 1998).

Spiders are one of the largest taxa on Earth, with almost 47,000 described species in 113 families (World Spider Catalogue 2017). Spiders regulate their behaviours relying on circadian clocks (Seyfarth 1980; Schmitt et al. 1990; Suter 1993; Yamashita & Nakamura 1999; Ortega-Escobar 2002; Jones et al. 2011) and exhibit formation of daily rhythmical in behaviour and physiology (e.g., Kovoor et al. 1995, 1999; Yamashita & Nakamura 1999; Nørgaard et al. 2006; Jones et al. 2011). Many spiders are nocturnally-active, although some species among the Salticidae, Oxyopidae, Thomisidae and Lycosidae are diurnal (Foelix 1996; Cloudsley-Thompson 2000). The Salticidae, or jumping spiders, is a large family of diurnally-active cursorial spiders (Jackson and Pollard 1996; Nelson & Jackson 2011), with more than 6000 described species in more than 600 genera (Coddington & Colwell 2001; Maddison 2015; Prószyński 2017). This family is an excellent candidate for investigation on temporal behaviour as they inhabit a variety of habitats, from sub-polar alpine regions to tropical rainforests.

In this thesis, I use locomotory activity measurements over several days to examine various aspects of temporal behaviour in different species of salticids. Chapter 2 is a series of comparative experiments using four salticid species to investigate the effects of temperature and light hours on activity patterns. For this, I changed the duration of light/dark cycle as well as temperature (to mimic seasonal changes) and I measure their activity over several days. Then I compare the responses of the different sexes and ages among these four species. In Chapter 3, I explore the relationship between external and internal non-photic drives on the locomotor activity of different salticid species. The specific aim of this study was to investigate the effect of non-photic drives on the activity of female *Marpissa marina (*Goyen 1892) and *Portia fimbriata* (Doleschall 1859). Here, I categorize the drives in two groups of manipulated and reproductive factors, with hungry, thirsty and differing food quality treatments

belonging to the former, and the reproductive factors being virgin spiders, mated spiders and spiders tested directly after laying eggs. Spiders, like the other small arthropods have wide surface-to-volume ratios and are at risk of losing water via evaporation (Chapman et al. 2013), which directly influences locomotor activity (Dethier 1976). In chapter 4, I investigate short-term temporal effects on *M. marina* behaviour across different light regimes simulating different latitudes. Specifically, I investigated how *M. marina* respond to shortening and lengthening of daylight hours by calculating the level and onset of activity over three successive days in a variety of L:D conditions. In Chapter 5, I describe the exact timing of locomotor activity in the salticid *Marpissa marina* with respect to LD cycles and then attempt to determine the mechanism by which this temporal phasing is controlled. For this, I employ the effect of total darkness (new moon), nocturnal dim light (emulating full moonlight), and intense (bright) light and 'normal' light on their activity. In chapter 6, I investigated Aschoff's rule using *M. marina* in DD and in constant light at different intensities to determine the effect of different light intensities in the maintenance of their periodicity. Finally, in Chapter 7, in a series of eye covering experiments, I investigated which of the four pairs of eyes of *M. marina* is capable of entrainment.

Spiders were collected as juveniles and raised to the maturity. Unless otherwise stated, all test spiders were virgin, as preliminary experiments showed that mating and egg laying physiology affects activity. All test spiders were sated (one fly always remained in the cage) with 2 house flies (or spiders for *Portia*) a day before experiment or after each experiment. The spiders maintained in the plastic cages under LD 12:12 at lab and in 25º-27 º, under 4 rows of white LED lams (spectrum : 400 to 700 nm) for experiment and 65-70% humidity inside incubator. After each experiment, all the test tubes and corks were washed with 70% alcohol and water and autoclaved. The spiders were released into their habitat after three or four experiments.

All means (averages) throughout the text of this thesis are reported as SD. Figures are reported as medians and quartiles, or as the mean or log-transformed mean of data and SEMs. All data was checked for normality using Schapiro-Wilk tests. In cases where data could be transformed to conform with normality, I used a log transform. In cases where a log-transform did not normalise data non-parametric statistics were used for further analysis. Repeated measures ANOVA tests were used for comparing more than 2 groups of treatments with Bonferroni post-hoc tests. In non-parametric analyses I used Friedman tests with Dunn`s post-hoc tests.

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Chapter 2

The effect of photoperiod and temperature on the locomotory activity of four species of jumping spiders

Abstract

While the daily rhythm derived from circadian clocks enables animals to follow daily changes in their surroundings, the photoperiodic timer enables animals to anticipate seasonal changes, which are especially acute at higher latitudes. This study aims to investigate the effects of temperature and light on the locomotory activity patterns of four species of jumping spiders (*Marpissa marina* (Goyen 1892), *Trite planiceps* (Simon 1899), *Portia fimbriata* (Doleschall 1859), *Servaea incana* (Karsch 1878)) by measuring their locomotor activity over 11 days in two photoperiods (long 14:10 LD and short 10:14 LD) and two temperatures (16° and 25°C). The results show a variety of responses in females, males and juveniles of spiders in different light/temperature conditions which reveal different sensitivity to light and temperature in different species of tropical and temperate jumping spiders.

Keywords: Locomotory activity, jumping spiders, photoperiod, temperature

Introduction

Two main rhythms have been recognized in animal's behaviour; a rhythm of daily activity (24 h) and an annual rhythm (12 mo) (Bradshaw & Holzapfel 2010). The daily (circadian) rhythm, derived from circadian clocks, enables animals to follow daily changes in their surroundings, while the photoperiodic timer enables animals to anticipate seasonal changes (Bradshaw& Holzapfel 2010), which are particularly acute at higher latitudes. Circadian rhythms can be reset (entrained) to dawn and dusk daily, while photoperiodism acts as a go/no-go seasonal switch that leads to animal's migration, dormancy, development, or reproduction (Pittendrigh 1960, 1965, 1981b; Aschoff 1965; Nijhout 1994; Bradshaw & Holzapfel 2007a). In response to seasonality, organisms from fungi (Roenneberg et al. 2010) to mammals (Kriegsfeld & Bittman 2010) have evolved mechanisms to synchronize with day length changes by using photoperiodic 'clocks' to measure and respond to changes in day length (Saunders 2002). Responses to seasonal changes are often very dramatic; for example, many

arthropods respond to decreasing daylight hours through diapause (Saunders 2002; Nishizuka et al. 1998; Koštál 2011; Saunders & Bertossa 2011), while others, such as spiders, go into a dormancy or torpor phase.

Current evidence suggests that almost all photoperiodic mechanisms are based on the pre-existing circadian 'clock', which acts as a pacemaker (reviewed by Hazlerigg 2011; Kriegsfeld & Bittman 2010; Putterill et al. 2010; Ronnenberg et al. 2010), as initially proposed 75 years ago by the German plant physiologist Erwin Bünning (Bünning 1936), but precise details on how these work are still not clear. Pittendrigh (1972) suggested the 'internal coincidence' model, which posits that separate 'dawn' and 'dusk' (morning and evening) oscillators are involved in measuring photoperiod, with seasonal changes in day length being measured as phase differences between the two, and light having the sole role of entrainment. Pittendrigh (Pittendrigh & Minis 1964; Pittendrigh 1966) also suggested the 'external coincidence' model, which includes a single oscillator entrained by the light/dark cycle, such that a particular light-sensitive phase falls in the latter half of the night. In the long nights of autumn, this phase falls in the dark, leading to behavioural and physiological changes, such as the induction of diapause or winter dormancy. In contrast, in short summer nights, the dawn transition of the photo-phase extends 'backwards' to illuminate the phase, thus inducing changes in activity, development and reproduction associated with warmer weather. In this model, light has two roles: 'entrainment' of the circadian oscillation(s) to the light-cycle and 'photo-regulation' of the alternate non-dormancy or dormancy-associated pathways by illumination or non-illumination of the photo-inducible phase (Saunders & Bertossa 2011).

In addition to light, low temperature and even restricted food may affect the photoperiodic timer (Saunders 1966a, b), and may be especially important among species that live in the tropics, where changes in daylight are minimal throughout the year, so photoperiod may be a less informative predictor of impending climate than in temperate regions. In several species of tropical flesh flies, induction of diapause is temperature dependent, whereas diapause in temperate species is induced by photoperiod (Denlinger 1974). Similarly, studies on several related species of mice suggest that mechanisms of photoperiodic and regulation of reproduction are mediated by different physiological mechanisms, which are affected both by phylogeny and environmental (latitudinal) factors (Trainor et al. 2006).

This study aims to investigate the effects of temperature and light on the locomotory activity patterns of spiders, a group in which this area of research has been understudied. The sole related study was on the nocturnal wolf spider *Lycosa tarentula* (Ortega-Escobar et al. 1992), in which adult females in a natural light/dark (LD) cycle altered the onset of their locomotor activity based on the time of sunset. Here, I do a comparative study on four species of jumping spiders (Salticidae).

The Salticidae is a large family of diurnally-active cursorial spiders, with more than 6000 described species in more than 600 genera (Coddington & Colwell 2001; Maddison 2015; Prószyński 2017), spread through a diverse array of habitats, from sub-polar alpine regions to tropical rainforests. Instead of a web, salticids, which are active, visually-based, hunters, typically build silken retreats or nests in which they rest (Boulton & Polis 2002; Wesolowska & Haddad 2002; McGinley et al. 2016; World Spider Catalogue 2017). I used four species in this comparative study. *Marpissa marina* (Goyen 1892) and *Trite planiceps* (Simon 1899) are temperate species from the South Island of New Zealand. *Marpissa* is found on rocky beaches around the pebbles or under beach debris, while *Trite* lives in the cavities formed by rolled-up leaves of New Zealand flax (*Phormium tenax*) and similar plants (Taylor 1998; Taylor & Jackson 1999). *Portia fimbriata* (Doleschall 1859) is from tropical forests in Queensland, Australia, where it builds very rudimentary webs in which it rests, but leaves to hunt its prey (Jackson & Hallas 1986; Jackson & Wilcox 1990; Chang & Tso 2004). *Servaea incana* (Karsch 1878) is found from subtropical to temperate regions on the East coast of Australia, where it lives under loose bark on *Eucalyptus* trees (Żabka1991; McGinley et al. 2016). The different habitats, climactic, and latitudinal zones of these four species permitted me to investigate the following questions:

- Are there any differences in activity level between species, and can these be attributed to the climactic region (or latitude) in which they occur?
- Based on the above, are there differences in activity levels at different temperatures or photoperiods? (i.e., is one of these factors a cue to seasonal change?)
- Do activity levels and timing differ depending on the time of day and on the sex or age class of the spiders?

Methods

24 field-collected (as juveniles) (8 virgin females, 8 males and 8 juvenile) *Marpissa marina*, *Trite planiceps* and *Portia fimbriata* ($n = 24$) as well as 11 individuals (4 virgin females, 4 males and 3 juvenile) of *Servaea incana* ($n = 11$) were used for this experiment. Due to mortality, we were unable to obtain data from *Servaea* for the short photoperiod testing, so only 14:10 data will be presented for this species, where relevant. For maintenance, spiders were individually-housed in plastic containers (40 mm diameter × 50 mm) with a ventilation hole covered with mesh and a hole at the bottom containing a cotton wick which hung into a glass of water to provide humidity and drinking water (Jackson & Hallas 1986). An additional hole, plugged with a cork, was used for feeding the spiders (with house flies, *Musca domestica* L.) once per week.

All spiders were fed to satiation on house flies 24 h prior to the experiment (i.e., there were always live prey remaining in the cage after 24 h). For testing, spiders were transferred individually into glass tubes (outer measurements, 16 mm diameter × 100 mm long; TriKinetics, Waltham, MA, USA) to measure locomotory activity. These were enclosed at one end by a snug-fitting small glass tube containing water plugged by a cotton wick that was inserted 10 mm into the locomotory activity tube in order to provide spiders with water throughout the experiment. Locomotory activity tubes were enclosed at the other end by mesh held by a rubber band.

Activity tubes were loaded into specially-designed locomotory activity monitors (LAM) (LAM25, TriKinetics, Waltham, MA, USA) enabling simultaneous recording for up to 32 channels (individual tubes). As each spider moved back and forth in its activity tube, it interrupted one of three infrared light beams that bisected the tube. Each crossing was counted by the system and the activity counts per min for each tube were sent through an interface unit (PSIU9, TriKinetics, Waltham, MA, USA) via USB to a computer. Data were pooled into 30 min bins using the dedicated activity monitoring software (DAM File-Scan, TriKinetics, Waltham, MA, USA) for further analysis. Monitors were housed inside environmental chambers (Contherm-POLAR1000, 50 cm high X 50 cm wide X 35 cm deep; internal diameters) at a controlled temperature (25^{o} C). Light was provided by a series of four strips of 12 wide-beam LED lights purpose-built for the chamber and placed on the inner ceiling to provide consistent lighting throughout the chamber. For this study, light was set at 500

lux as a usual light (neither intense nor dim), as measured in the middle of the chamber where the monitors were placed.

For individuals of each species (except *Servaea*, as noted above), I ran each experiment over 11 days in two photoperiods (long LD 14:10 and short LD 10:14) and two temperatures (16° and 25°C), resulting in four treatments. Females, males and juveniles of each species, were exposed to each treatment for 11 days, such that I had 24 replicates for each photoperiod/light combination in each species (except *Servaea*). Following the feeding procedure, I let them to rest for 3 days under a normal cycle of LD 12:12, and then I used the same spiders for next experiment (under the next treatment). The only difference was rearranging the spiders in the LAM machine in random order. Prior to testing, all spiders had been maintained on a 12:12 cycle. The first two days of testing were ignored to ascertain data for entrained spiders only was used. In the tests of onset and offset of activity, in all our experiments the "time-on" was considered as 7:00 and the "time-off", depends on the summer or winter daylight hours went to 21:00 or 17:00 respectively. Monitors were housed inside environmental chambers with controlled temperature and light. These could be controlled from outside to vary light intensity within the chamber. The spiders were fed after 5 days during light condition. All spiders were returned to the channels within the LAM in which they had been previously placed.

To analyse the data, the activity levels in all sexes and species of jumping spiders were collected in 30 min time bins and calculated as log mean activity levels (to normalize the dataset) and the average daily activity for the 11 days of the experiment was analysed using RStudio (Version 0.99.903). Data were further analysed using one-way repeated measures ANOVA, with Bonferroni post-hoc comparisons where applicable. Data were graphed in Prism and Excel. Onset and offset of activity and length of the active phase through the day was coded into R (Rjags, Dplyr, Ggplot and Ggvis packages).

Results

All results are reported as the mean number of interruptions of the IR lights, indicating mid-tube crossings ± SD in each 30 min time bin over 11 days of testing.

Combining all light and temperature treatments, *Portia* was the most active species, with *Marpissa* and *Trite* being less active and similar to each other ($F_{2,46}$ = 34.71, P <0.05, *N*=24) (Fig. 1). This trend was also evident when data were divided into different temperature treatments (Fig. 2). Here, there were differences in activity levels according to the sex/age groups, but these did not follow consistent patterns across species. In *Portia* ($F_{2,14}$ = 15.47, P < 0.05, *N*=8), females were the most active group, with males and juveniles having similar activity levels, while among *Marpissa* ($F_{2,14}$ = 15.29, P < 0.05, *N*=8), juveniles had significantly lower activity levels than males and females. In *Trite* ($F_{2,14}$ = 4.52, P = 0.05, *N*=8), juveniles were the most active group (Fig. 2). Trends followed a very similar pattern in regard to different day-light length when data for 25° & 16° were combined (LD 14:10: *Marpissa,* $F_{2,46}$ = 10.02, P < 0.05, *N*=24; *Portia*, $F_{2,46}$ = 18.82, P < 0.05*, N*=24; *Trite,* $F_{2,46}$ = 2.88, P = 0.080*, N*=24; LD 10:14: *Marpissa,* $F_{2,46}$ = 7.53, P = 0.002*, N*=24; *Portia*, $F_{2, 46}$ = 3.055, P = 0.061*, N*=24; *Trite*, $F_{2, 46}$ = 1.82, P = 0.179*, N*=24; Fig. 2).

To normalize activity by 'time of day', data during daylight hours were divided into five equal time bins (2 or 2.8 h, for 10 and 14 h 'days', respectively), representing morning, midmorning, midday, afternoon and evening (Fig. 3). These data generally show that all species had slightly higher activity levels during LD14:10 photoperiods. *Marpissa* had similar activity levels at different temperatures during long photoperiods, while in short 10:14 photoperiods, activity in colder levels was lower throughout the day. Irrespective of photoperiod, in all other species, activity levels for each sex/age group showed differences at the different temperature treatments, with activity being higher at warm temperature for some groups (e.g., female *Portia*) but lower for most groups in most species (Fig. 3). As a typical pattern, activity levels tended to be highest during the middle of the day and afternoon, and reduced considerably in the evening. Data for the shorter photoperiod for *Servaea* are missing due to spider mortality, but during the longer photoperiod, this species consistently had higher activity levels at all sex/age groups in the morning at both cold and warmer temperatures.

When data are plotted over 24 h, *Servaea* and *Marpissa* are notable in having a distinct peak of activity in very first hours of darkness, which is also, to a limited extent, true of *Trite* (Fig. 4). Additional details about activity differences between species can be gleaned by looking at the onset of activity in different seasonal photoperiods and temperatures (Fig. 5). Overall, *Servaea* and *Marpissa* started their activity latest with

respect to lights-on, with *Trite* spiders starting their activity with a small delay while in *Portia,* activity began before lights on (Table. 1). At 25°, all sex and age groups (Fig. 5) except for *Servaea* (where males started activity only at the end of the day) typically began moving about roughly at daylight onset, except for all groups of *Marpissa,* which began moving before lights on. In contrast, at 16° *Marpissa* were typically delayed in their activity patterns, which was also true to some extent in *Servaea* and *Portia* (the latter only in the shorter photoperiod). There was considerably more variation in the dropping off of activity levels (offset of activity), with *Portia* and *Trite* tending to stop activity well before lights off, irrespective of photoperiod or temperature (with the exception of juvenile *Trite* at lower temperatures). In contrast, *Servaea* ended activity before lights-off only at colder temperatures. The final clear pattern emerging from these data is that females tended to reduce their active period at lower temperatures (Fig. 5). A comparison of mean onset of activity showed that juveniles were typically the earliest to move about, followed by males and then females in *Portia, Marpissa* and *Trite* (although this difference was minimal among *Portia*), while in *Servaea*, females were followed by juveniles then males. Finally, spiders were active longer at 25º rather than 16º; there is a direct relationship between the delay in onset of activity and the length of the active day in all species (Fig. 6).

Table 1. The mean latency/advance in onset of activity + SD towards light-on time, in *Marpissa*, *Portia*, *Servaea* and *Trite* spiders.

species	Mean (h)	SD (h)	*N*
Marpissa	1.88	1.01	24
Portia	-0.12	0.53	24
Servaea	3.61	1.53	11
Trite	0.61	0.48	24

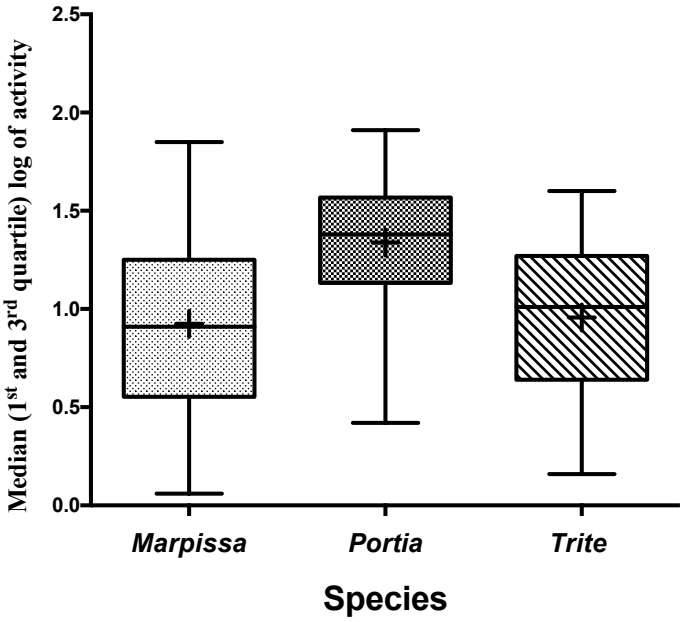

Fig. 1. Box-plots of median value of activity for each species across all treatments during 30 min time bins over entire days of experiment. Error bars drawn based on min and max. Crosshairs represent mean activity level.

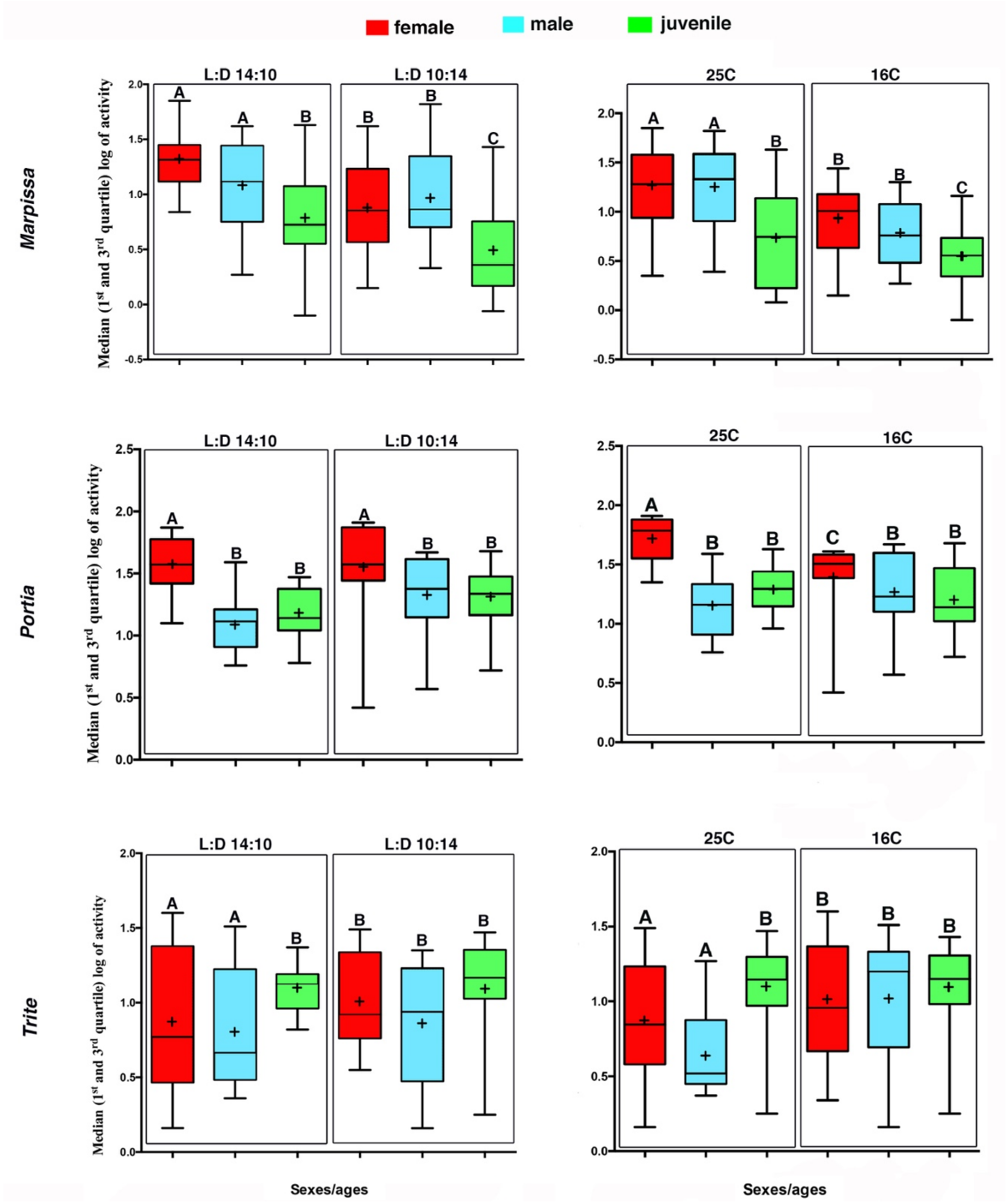

Fig. 2. Box-plots of median value of activity of different sexes/ages for each spider species during 30 min time bins depicting data obtained in 'long light/dark (LD 14:10) and 'short' (LD 10:14) cycles, irrespective of temperature (left panels) and obtained in 'cold' (16°C) and 'warm' (25°C) conditions, irrespective of daylight hours (right panels). Error bars drawn based on min and max. Crosshairs represent mean activity level. The letters (A,B and C) show significant differences at alpha = 0.05 (Bonferroni test).

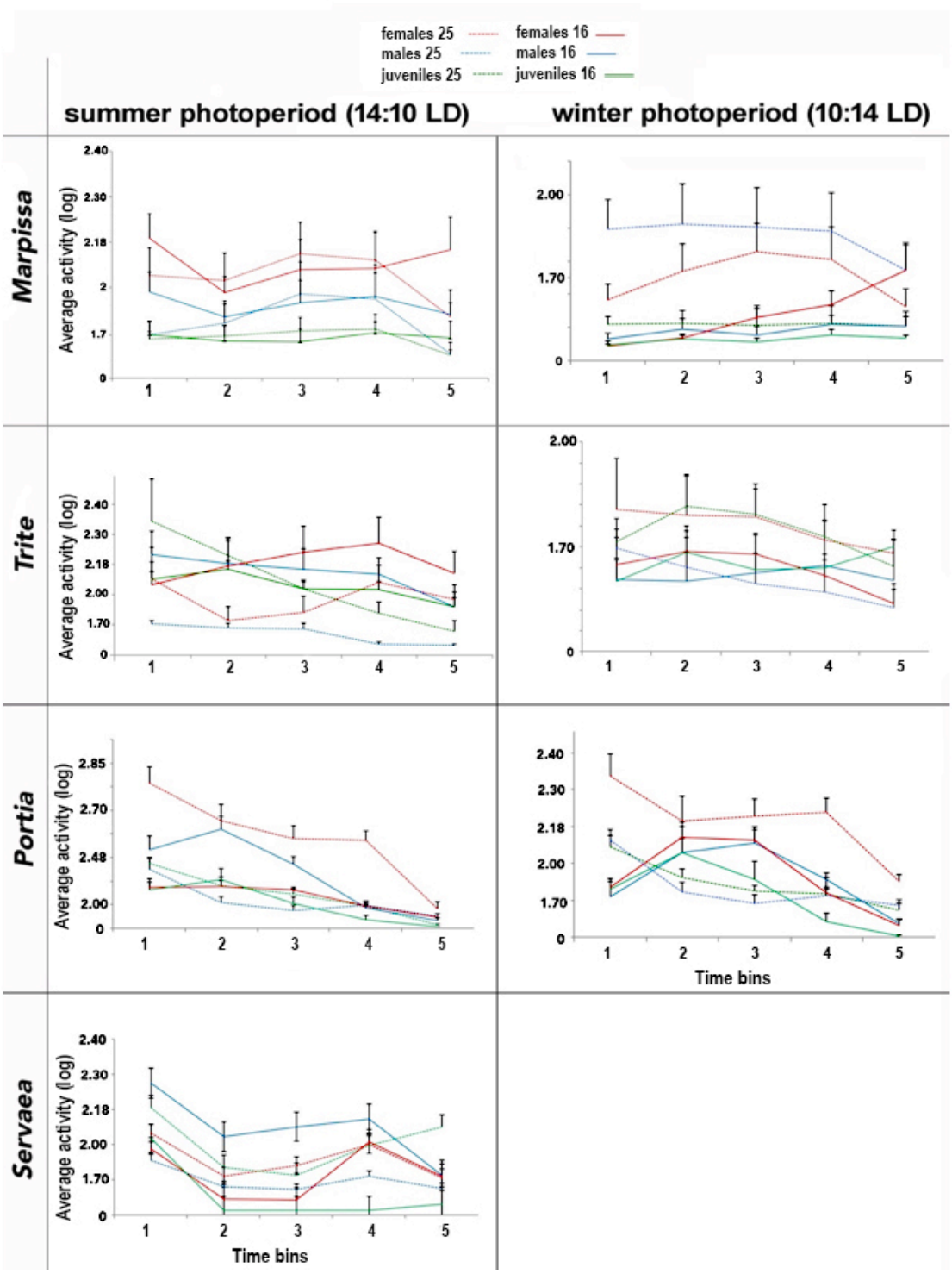

Fig. 3. Average activity (SEM) over five daily time periods (representing morning, midmorning, midday, afternoon and evening to normalize activity by daylight 'time of day' irrespective of photoperiod), for all spider species under LD 14:10 and 10:14 at 16° and 25°C for all sexes/ages.

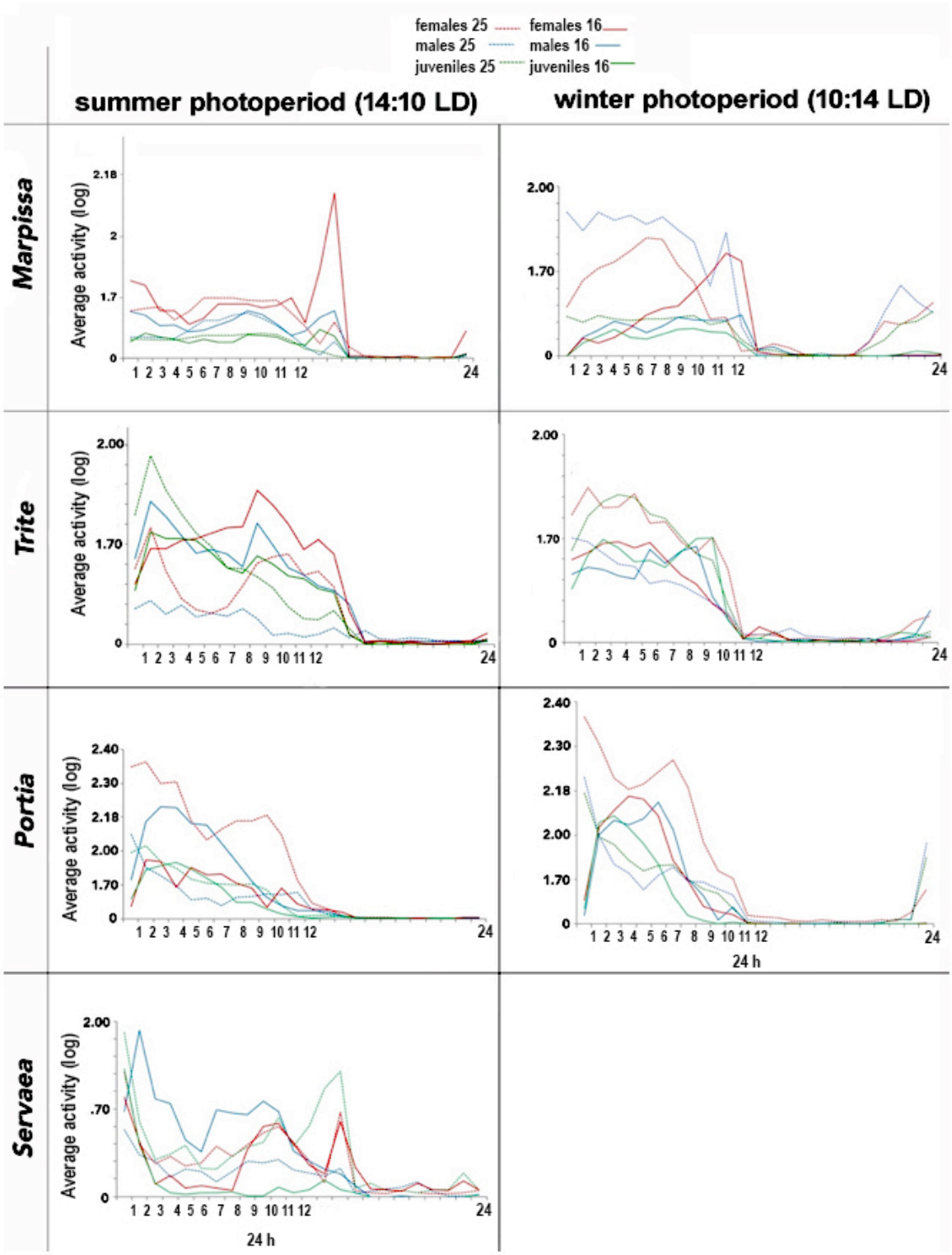

Fig. 4. 24 h activity plots of females, males and juveniles of all spider species under long (summer) photoperiod of LD (light/dark cycle)14:10 and short (winter) photoperiod of LD 10:14, in cold (16°) and warm (25°) conditions. Note: Data for the shorter photoperiod for *Servaea* are missing due to spider mortality.

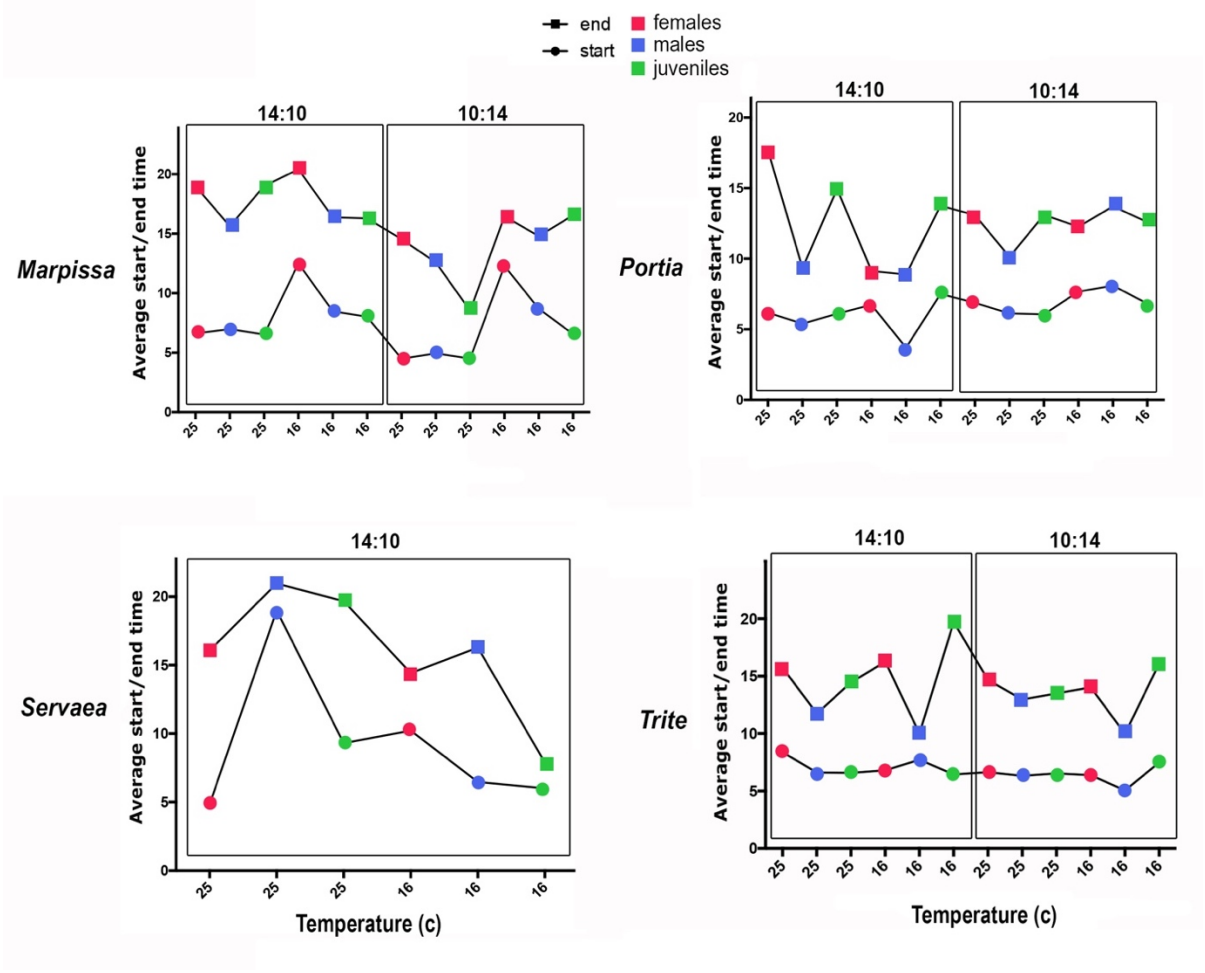

Fig. 5. Average onset (circles) and offset (squares) of activity (based on light-on time at 7:00 am) in all species (*Marpissa, Portia, Servaea* and *Trite*) (all sexes and ages) in different photoperiods, under cold and warm temperature. Under LD (light/dark cycle) 14:10, lights-off happened at 21:00 and under LD 10:14, it was off at 17:00.

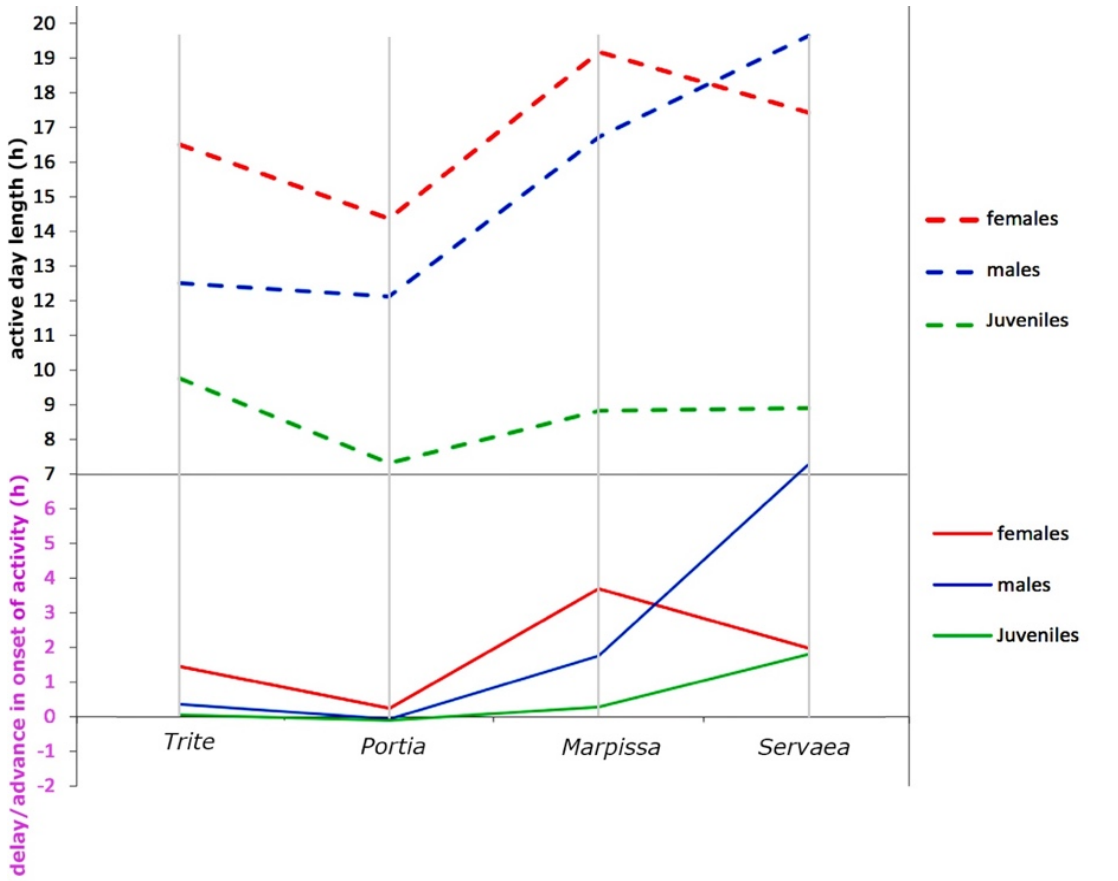

Fig. 6. Representation of the delay in onset of activity in all sexes and ages of salticids (below) and their period of activity (above).

Discussion

My results showed clear differences in activity levels in the four species for which data was obtained in all treatments, with *Portia* being the most active species. *Portia* is a tropical species and the other two species are temperate, but there is not enough evidence to suggest that differences in activity can these be attributed to the climactic region or latitude in which they occur, even though *Marpissa* and *Trite* had similar overall activity levels and are from the same region in New Zealand. While these results are suggestive that they may be characteristics of circadian organization that are linked to latitude, it is known that the degree of circadian organization between species differs widely depending their biology (Bloch et al. 2013). For example, in a comparative study on the sociability and reproductive behaviour in five related species of mice, it was found that latitude of origin had no consistent effects on responsiveness to photoperiod (Trainor et al. 2006).

The alternative hypothesis that other aspects of their biology may accounts for across species differences in activity can not be ruled out. For example, *Portia fimbriata* is a highly unusual salticid which has a specialized predatory behaviour for hunting spiders, which are dangerous prey to tackle (Jackson & Hallas 1986). *Marpissa* and *Trite,* on the other hand, hunt insects, like most salticids do (Jackson & Tarsitano 1993), so it could be argued that araneophagy (spider-eating) requires higher levels of activity – an argument which could be supported by my finding that female *Portia* were the most active, and females are known to be more voracious. However, females are the voracious sex in salticids generally (Buskirk et al. 1984), and the females of other species, such as *Servaea* and *Marpissa* (at low temperatures) were not more active than other sex/age classes, although generally the pattern of high female activity held.

I was also interested to determine whether there are species-specific and sex or age class differences in activity levels at different temperatures or photoperiods, such that one of these factors might be a cue to seasonal change. Circadian activity patterns differed widely between species in this study and, within species, also differed depending on both temperature and photoperiod. It is known that the expression of circadian patterns can differ within a species depending on sex. For example, mouse lemurs have sex-specific responses to photoperiod regarding synchronization of the breeding season

(Perret & Aujard 2001). In this study, there were fairly consistent sex/age class differences between the groups. In *Marpissa,* regardless of photoperiod or temperature, females had the highest activity and juveniles the lowest. This pattern was not dissimilar among *Portia,* but very different in *Trite*, where males and females had similar, and lower, levels of activity than juveniles. High levels of activity in females and in juveniles is consistent with hunting, as these are the two groups where foraging is a high priority. Male spiders typically do not hunt often, instead searching for females (Bell 1990) - an argument could thus be made for males to have high levels of activity, especially in temperature species with a reduced breeding window. However, my results do not show a clear pattern in this regard.

Generally, salticids started their day earlier in summer and later in winter and there was less variation among juveniles than adults. *Portia* tended to be active primarily in the mornings, while *Trite* and *Marpissa* tended to have a fairly uniform level of activity throughout the day and *Servaea* was biphasic, with peak activity in the morning and late afternoon. As expected, spiders were active for shorter durations during the cold, winter photoperiod, due to both becoming active later in the day and ending their activity earlier, well before 'sunset'. The treatment in which there were most hours of activity was in the warm summer photoperiod (14:10 LD, 25°), with spiders often using many available daylight hours in active pursuits, suggesting that these are ideal conditions for searching for food or mates. Similarly, lycosid spiders, *Lycosa tarentula*, also track seasonal differences in sunset; as nocturnal spiders, their activity begins earlier when sunset is earlier (Ortega-Escobar et al. 1992).

I expected that, particularly in temperate species, there would be a drop in activity associated with a shorter photoperiod and/or colder temperatures as in species of insects which have low temperature optima for diapause, the combination of day length and warmth in summer, prevents them entering diapause, but as soon as the temperature drops below a threshold, they enter a phase of dormancy and reduction in activity (Tauber & Tauber 1976). In jumping siders, while the hours of activity were reduced, I found mixed evidence for a specific lessening of movement. In the 10:14 shorter photoperiod, *Marpissa* was less active at cold temperatures, which was also true in *Trite*, but less obvious in *Portia*. In the longer 14:10 photoperiod, sex and age class differences were less consistent, although all groups were generally more active in

longer days than in the shorter photoperiod, irrespective of temperature. This suggests that behavioural changes were primarily associated with photoperiod rather than temperature, even in the tropical species, *Portia*. This is consistent with Tanaka's (1992) results on both temperate and subtropical populations of the house spider *Achaearanea tepidariorum* having photoperiodically-induced periods of dormancy (diapause). Nevertheless, it is worth noting that all species except *Portia* tended to ramp up activity later in the day during the 10:14 photoperiod compared with the 14:10 photoperiod; in *Portia* this only happened at the colder temperature, and irrespective of the photoperiod, suggesting that this species was more sensitive to thermal changes than the temperate species. Overall, these results suggest that the temperate species studied here were more sensitive to photoperiod than temperature and the tropical species, *Portia*, was sensitive to both photoperiod and temperature. These results are entirely consistent with the notion that low temperature is not, in and of itself, a cue but rather acts through developmental rate to reinforce the effect of short days in inducing diapause, as suggested for typical temperate species (Saunders 2002; Danks 2005).

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Chapter 3

The influence of non-photic biological drives on the locomotor activity of jumping spiders

Abstract

Locomotor activity is the most widely used measure to ascertain an animal's response to ambient changes. While light/dark cycles are known to be of primary influence to circadian rhythms, non-photic effects interact with the circadian cycles. This study aims to investigate the effect of non-photic drives on the locomotor activity of female *Marpissa marina* (Goyen 1892) and *Portia fimbriata* (Doleschall 1859). The drives were categorized in two groups of manipulated and reproductive factors, with hungry, thirsty and differing food quality (in *Portia*) treatments, and the reproductive factors being virgin spiders, mated spiders and directly after laying eggs over nine successive days under LD 12:12. The results show a significant increasing in activity in thirsty spiders in both groups, but fed and hungry spiders were not different. The quality of the food influenced the activity of *Portia* females. Also, mated and virgin spiders (*Marpissa*) were not different, while spiders after laying the eggs reduced their activity dramatically.

Keywords: thirsty, hungry, egg-laying, food-quality, virgin, mating

Introduction

Locomotor activity is the most widely used measure to ascertain an animal's response to photic and other ambient changes (Page & Larimer 1972; Daan & Aschoff 1975; Rieger et al. 2003). While light/dark cycles are known to be of primary influence to circadian or biological rhythms through the effect on entrainment of the biological clock, non-photic effects often interact with the circadian cycle in determining the practical functioning of biological clocks. Until 1967, findings (Gwinner 1966; Menaker & Eskin 1966; Lohmann & Enright 1967) suggested that non-photic incidences may be weaker than light as clock regulators, or zeitgebers (as measured either through changed levels of activity, for example, or through the proportion of individuals responding to the zeitgeber; Reebs & Mrosovsky 1989), but it is now

thought that this is species-dependent. For instance, Aschoff (1989) suggested that, for people, social entrainment is more important than light, but evidence of social entrainment on circadian activity in other mammals is conflicting (Erkert & Schardt 1991; Refinetti et al.1992). However, there are several possible non-photic zeitgebers, both external to the animal and internal, including sexual arousal (Honrado & Mrosovsky 1989), and stress. Few needs (if any) are more essential than food and water on a daily basis as the philosopher Bacon quotes from Seneca: "The desires of the body are few: relief from cold, hunger, and thirst" (from Jourjine 2017). Hunger and thirst are key links between ingestion and metabolic need (Berridge 2004) although drinking and eating are qualitatively different behaviours, and hunger and thirst are different internal experiences (Jourjine 2017), and thus not directly measurable in animals. Like sexual arousal, and fear and aggression, hunger and thirst are biological 'drives' that entail behaviours oriented into a particular goal (Jourjine 2017), but how these drives work is not entirely clear. For instance, Lorenz & Leyhausen (1973) suggested that a key aspect of drives is to gradually increase in intensity until the animal shows a specific reaction, while others (e.g., Tinbergen 1951) have demonstrated diverse interactions across different drives.

Small arthropods like spiders have wide surface-to-volume ratios and are at considerable risk of losing water via evaporation (Chapman et al. 2013), which can directly affect locomotor activity (Dethier 1976). However, food and water deprivation may have different effects on the activity of different animals. The interdependence of food and water deprivation has been studied extensively in vertebrates, including dogs and rats (Hall 1956; Cizek 1959; Bolles 1961), but is less well studied in invertebrates. In blowflies *Phormia regina* (Browne & Evans 1960), locusts *Locusta migratoria* (Edney 1937) and in *Drosophila melanogaster* (Connolly 1966), food deprivation causes increases in general activity. In contrast, Reynierse et al. (1972) demonstrated that hunger and thirst decrease cockroach (*Nauphoeta cinerea*) activity, with water availability being more important than food. Campbell & Sheffield (1953) believed that increased 'drive' through food deprivation does not in itself instigate activity, but instead lowers the threshold for other stimuli to instigate activity.

The effects of reproductive status on activity patterns have been relatively

poorly studied. Several studies have reported changes in activity levels in reproductive females. For example, virgin female and male *Drosophila melanogaster* have similar activity levels, but mated and pre-egg-laying *D. melanogaster* are more active than males, possibly because they are searching for a site to lay eggs (Isaac et al. 2009), whereas ants reduce activity (Bernadou & Heinze 2013). Also, Harano et al. (2007) showed egg-laying bee queens have reduced locomotor activity compared to virgin queens.

The term "araneophagic salticids" refers to jumping spiders (Salticidae) that specialise on eating other spiders (Richman & Jackson 1992). The most extensively studied araneophagic salticids are from the genus *Portia* (Jackson & Pollard 1996) and, in particular, *P. fimbriata* from Queensland. Jackson (1992) showed that *Portia* chose salticids in preference not only to insects but also to other spiders as prey. In this study, I was interested to understand the relationship between external and internal non-photic drives on the locomotor activity of different species of jumping spiders. The specific aim of this study was to investigate the effect of non-photic drives on the locomotor activity of female *Portia fimbriata* and *Marpissa marina*. The drives were categorized in two groups of manipulated and reproductive factors, with hungry, thirsty and differing food quality treatment belonging to the former, and the reproductive factors being virgin spiders, mated spiders and spiders tested directly after laying eggs. As predators are expected to express prey-preference primarily when prey is abundant (Jackson & Olphen 1991, 1992), I was interested to know how differences in prey type can influence the locomotor activity of spiders. For this, I used females of *P. fimbriata*, as female spiders are more voracious than males (Vollrath & Parker 1992). *Marpissa marina* is a generalist species, but Harland & Jackson (2001) reported strong stalking behaviour in females towards flies and these are evidently preferred over aphid prey (Moss et al. 2006) so, here, I used a fed treatment of flies for *Marpissa* to compare with hungry spiders. The most important objectives of this chapter were:

- Do physiological drives affect the activity level of spiders?
- Do physiological drives change daily patterns of activity in spiders?
- Do females from different species of jumping spiders behave differently when food and water deprived?

Methods

Sixty-three field-collected (as juveniles) females of the New Zealand temperate salticid, *Marpissa marina* (Goyen 1892), and 55 females of the Australian tropical salticid, *Portia fimbriata* (Doleschall 1859), were used for the series of nine-day experiments. The experiments were divided into two categories of manipulated drives and reproductive drives. For the reproductive drive experiment, I used 11 individuals of *Marpissa* for each of three treatments: adult virgin, mated, and one to two days after egg laying (these females were removed from the nests in which they laid their eggs). For the manipulated drive experiments, I tested *Marpissa* and *Portia*. For *Marpissa*, 11 females (N = 11) for each of three treatments were used. The treatments were thirsty spiders, hungry spiders, and a control group given access to food (*Musca domestica*) and water. For *Portia,* 11 individuals (N = 11) for each of five treatments were used. The treatments were thirsty spiders (fed on flies, but without access to water), hungry spiders (just given access to water), spiders fed on flies (*Musca domestica;* low quality food, as well as access to water), spiders fed with spiders (*Badumna longinqua* (Desidae) from New Zealand*;* high quality food, as well as access to water). The treatment of fly-fed spiders was considered the control group. For both species, spiders (except in hungry treatments) entered the experiment six hours after feeding (as preliminary experiments showed their activity is reduced in first few hours after feeding). For control groups and in *Portia* food quality treatments, spiders were feed on day four in their cages (during light hours) and were returned to the testing tubes after 6 h (still during light hours) to resume the experiment. Hungry treatment spiders entered the experiment three days after their last feeding. Thirsty spiders started the experiment after one-day of water deprivation and as the control treatment. Except for the thirsty treatment, all spiders had access to water throughout the experiment, which was carried out over nine successive days under LD 12:12.

For maintenance, spiders were individually-housed in plastic containers (40 mm diameter × 50 mm) with a ventilation hole covered with mesh and a hole at the bottom containing a cotton wick which hung into a glass of water to provide humidity and drinking water (Jackson & Hallas 1986). An additional hole, plugged with a cork, was

used for feeding the spiders with house flies, *Musca domestica* or with *Badumna longinqua*. The methods of locomotor activity measurements were as described in Chapter 2 and only details specific to this study will be described here. For testing, spiders were transferred individually into glass tubes (outer measurements, 16 mm diameter × 100 mm long; TriKinetics, Waltham, MA, USA) to measure locomotory activity. Except for the thirsty treatment, these tubes were enclosed at one end by a snug-fitting small glass tube containing water plugged by a cotton wick that was inserted 10 mm into the locomotory activity tube in order to provide spiders with water throughout the experiment (in thirsty treatment, this side of the test tube was enclosed by a cork). Locomotory activity tubes were enclosed at the other end by a loose cork (Fig.1). Mean activity levels for each 30 min over successive days of the experiment were measured. Data were analysed in Prism using Friedman test and Dunn`s test was used as post-hoc test when comparing the treatments whenever differences were significant.

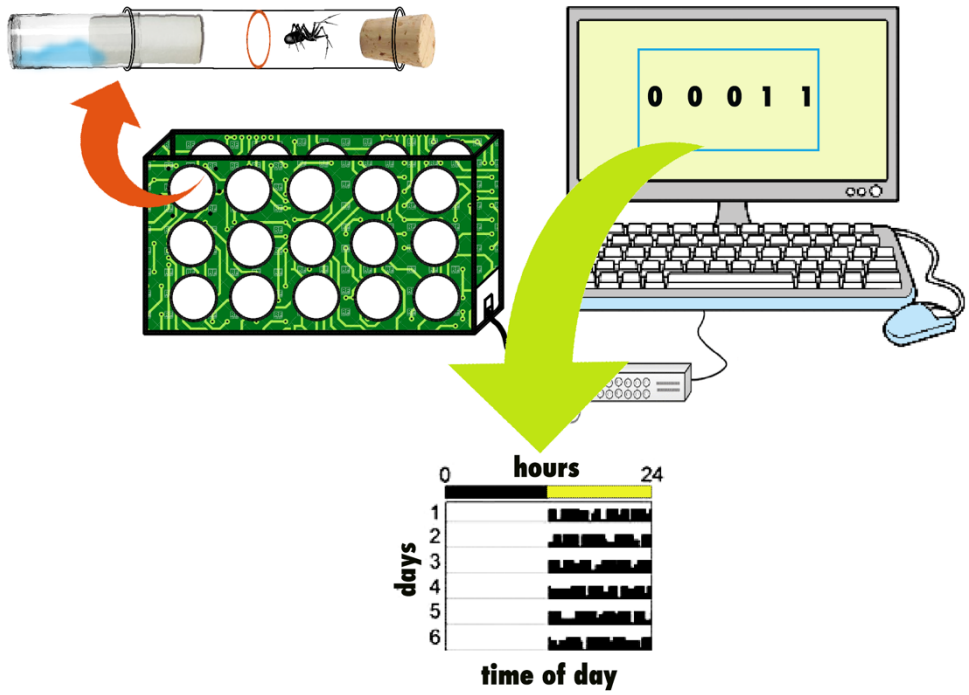

Fig.1. Activity measurement by means of DAM File-Scan (TriKinetics, Waltham, MA, USA). It contains monitor (s) with channels for setting test tunes which are stranded by infrared beams. The test tubes enclose by water-supplier tube at the one end and a loose cork at another end. After scanning the data by DAM software, the actograms are produced by Image-j (actogram-J) software for further study.

Results

For the manipulated factors, in *Marpissa*, there was a significant effect of treatment on activity levels (X^2_2 = 16.54, P = 0.0002, N=11) with thirsty spiders being significantly more active than the other groups (Fig. 2a). All groups showed similar trends in activity patterns, characterized by low activity at the start and end of the day and a peak of activity before lights-off (Fig. 2b). In *Portia*, there was also a significant

effect of treatment on activity levels (X^2_3 =27.13, P<0.00001, *N*=11), and Dunn`s post-hoc tests showed that females that fed on spiders and thirsty spiders were significantly more active than the other treatments (Fig. 3a). In almost all treatments, spiders showed the same trends in six daylight time bins, decreasing their activity dramatically before darkness under all conditions (Fig. 3b).

There was a significant effect of treatment on activity levels in terms of the reproductive factors (X^2_2 = 16.90, P = 0.00021, *N*=11). *Marpissa* females significantly reduced their activity after egg laying (to almost nothing), but there was no difference between the activity levels of virgin and mated spiders (Fig. 4a). All groups showed similar trends in activity patterns, characterized by a dip in middle of the day and a peak of activity before lights off (Fig. 4b).

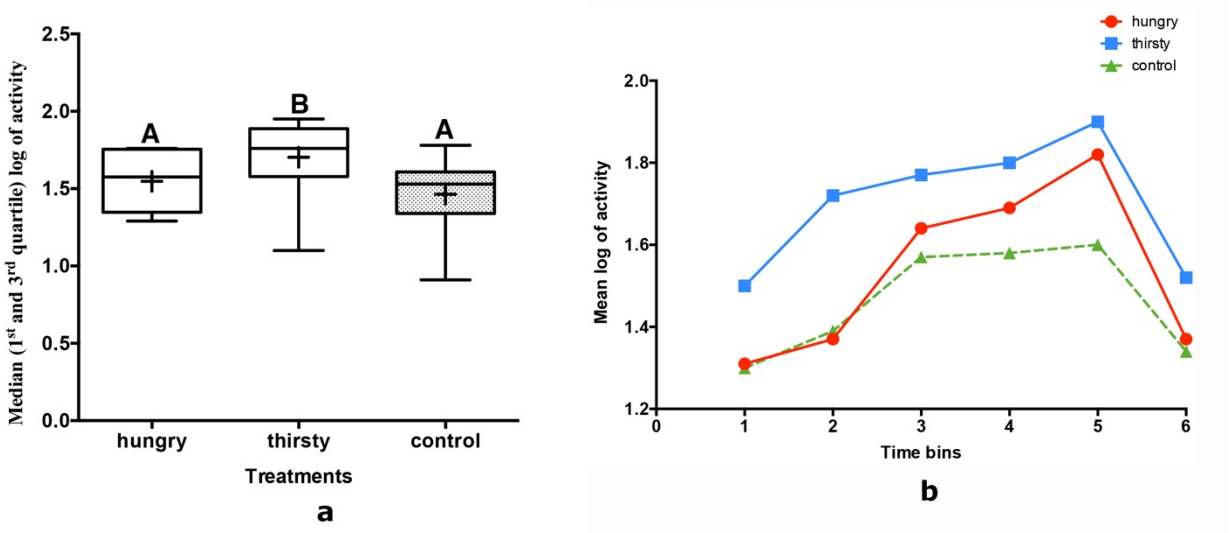

Fig. 2. Activity patterns of hungry and thirsty *Marpissa* females compared with controls. **a.** Box-plots of median value of activity of different treatments. Error bars drawn based on min and max and mean logarithm of activity for each group depicted by "+". Different letters denote significant differences at alpha = 0.05, Dunn`s test. **b.** Average activity over six 12:12 LD daily time bins (2 h each) for 6 cycles.

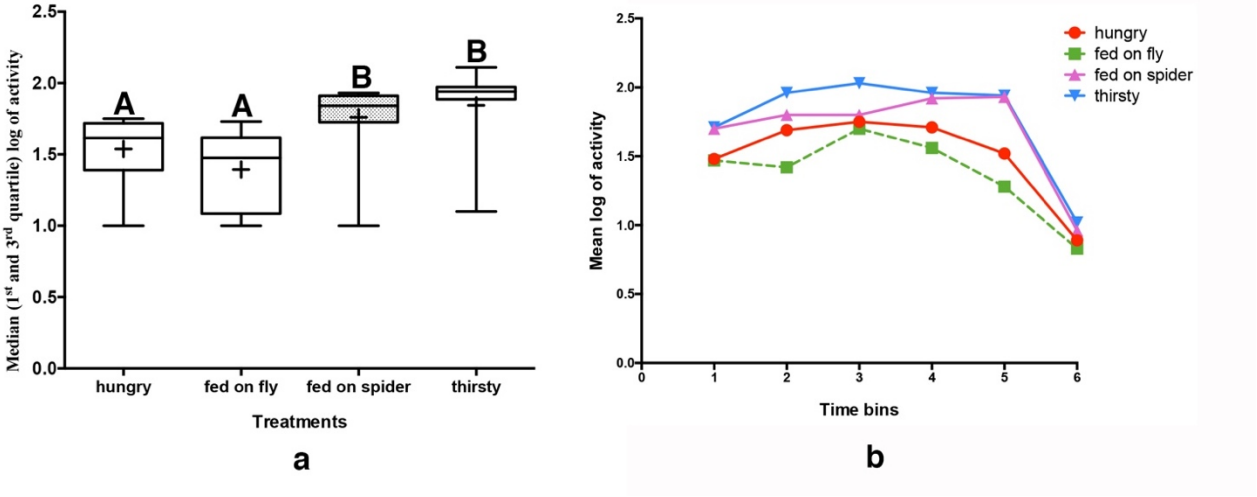

Fig. 3. Activity patterns of hungry, thirsty, spider-fed and fly-fed *Portia* females compared with controls. **a.** Box-plots of median value of activity of different treatments. Error bars drawn based on min and max and mean logarithm of activity for each group depicted by "+". Different letters denote significant differences at alpha = 0.05, Dunn`s test. **b.** Average activity over six 12:12 LD daily time bins (2 h each) for 6 cycles.

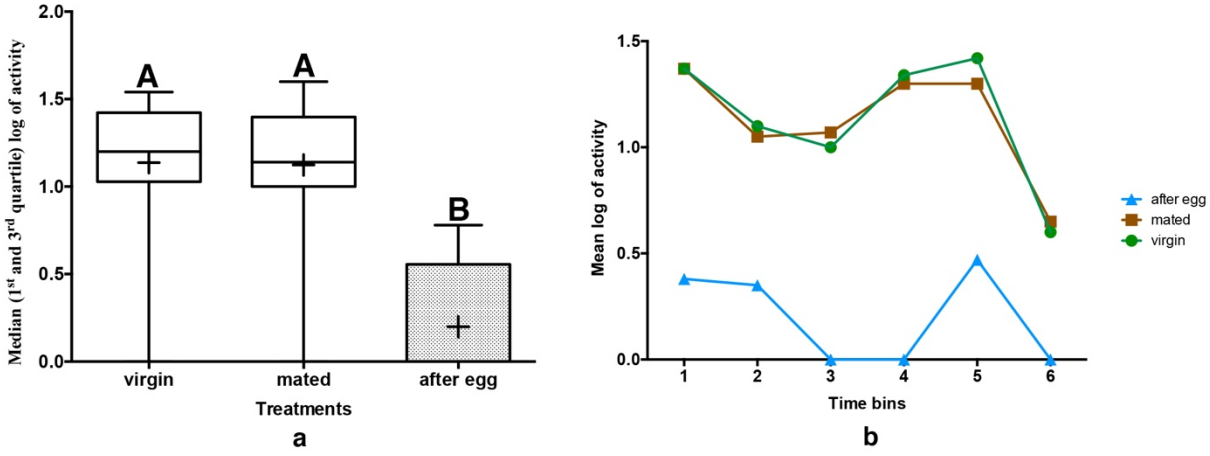

Fig. 4. Activity patterns of virgin, mated, and recently egg-laid *Marpissa* females. **a.** Box-plots of median value of activity of different treatments. Error bars drawn based on min and max and mean logarithm of activity for each group depicted by "+". Different letters denote significant differences at alpha = 0.05, Dunn`s test. **b.** Average activity over six 12:12 LD daily time bins (2 h each) for 6 cycles.

Discussion

Multiple factors affect activity levels in female salticids, but these same factors do not appear to affect the distribution of activity patterns throughout the day. Both salticid species showed anticipatory behaviour before darkness under all the treatments. Overall, my results show that, despite the influence of non-photic drives on the locomotor activity of salticids, the diel pattern of activity is retained irrespective of physiological or reproductive drives.

In both *Marpissa* and *Portia*, there was no difference between hungry spiders and those fed on flies, yet *Portia* fed on their preferred prey (spiders) were significantly more active, suggesting that for this specialist predator (Jackson 1992), spiders provides nutrition that permits spiders to be more active than they would be otherwise. It is known that *Portia* require spiders to survive to adulthood (Li et al. 1997), and in other specialist predatory spiders in the Zodariidae have been found to extract nutrients from their preferred prey which have beneficial fitness effects (Pekár et al. 2008). Several other studies investigating the effects of hunger in activity levels (e.g., Edney 1937; Hall 1956; Browne & Evans 1960; Connolly 1966; Provencher & Riechert 1991; Walker et al. 1999) have found that hunger leads to an increase in activity, while others (e.g., Reynierse & Cafferty 1972) have found that hunger decreases activity levels, but neither of these effects was found here. However, spiders are known to have a very low metabolic rate (Anderson 1970), so it is possible that the duration of my experiments was insufficient to cause major hunger-related stress in spiders that could be perceived through a change in activity levels. In contrast, in both *Marpissa* and *Portia*, thirsty spiders were more active than spiders that had water available to them, whereas in cockroaches, thirst decreases activity (Reynierse & Cafferty 1972). Usually, undernourished animals behave in such a way as to increase the likelihood of detecting and exploiting a resource. Because starvation affects both the advantage and cost of foraging, it has different influence on behaviour (Scharf 2016). In arthropods, usually a long term starvation causes an increase in activity, albeit also in some cases, because of the low metabolic rate or exhaustion, a decrease in activity may be possible (Scharf

2016). This suggests that in the short timeframe of these experiments, spiders were more strongly affected by lack of water than by lack of food.

Reproductive status strongly affected the activity level of female *Marpissa.* While there was no significant difference between the activity of virgin and mated spiders, spiders that had recently laid eggs became almost inactive. Aigaki & Ohba (1984) showed that the mating status of *Drosophilla virilis* affected the longevity of flies differentially depending on sex (virgin females lived longer and males had shorter lifespans), but did not investigate activity levels. Female and male *Drosophila melanogaster* have similar activity levels, but mated and pre-egg-laying *D. melanogaster* are more active than males, possibly because they are searching for a site to lay eggs (Isaac et al. 2009), whereas egg-laying ants (Bernadou & Heinze 2013) and egg-laying bee queens (Harano et al. 2007) have reduced locomotor activity. In salticids, mated females spin a thick silken nest in which they lay their eggs. Typically, females stay with their eggs until after they hatch and the spiderlings emerge from the nest (Yip & Rayor 2014). During this time, females rarely leave the nest to go and find food, so the low activity levels in this group are not entirely surprising. However, it is interesting that even in this group that had such low activity levels, the patterns of activity throughout the day were similar to the other groups.

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Chapter 4

Effect of latitudinal phase shifting and zeitgeber direction on activity patterns of jumping spiders (Salticidae)

Abstract

Photoperiodism relates to how animals assess the length of the day as an anticipatory key to time-related events in their life history and thus is affected by both latitudinal and seasonal changes. In addition to the latitudinal variation, response to changes in daylight hours may also be due to different clock mechanisms. Spiders appear to exhibit daily rhythmical facets in behaviour and physiology. I used virgin female *Marpissa marina* (Goyen 1892; Araneae: Salticidae) in sequential three-day parts of experiments with delay (6 h and 10 h) and advance(6 h) shift after entrainment cycle and after freerunning under constant dim light. In fact, playing with light-on time to stimulate moving through latitudes. I measured the locomotor activity and latency (and advance in onset of activity) in spider as the response to light-on time. Spiders showed a faster response to an advance in time-shift.

Key words: advance shift, delay shift, after effect, circadian clock, latency

Introduction

Circadian rhythms are endogenous biological oscillations with a period of about 24 h (Halberg 1958). An external signal acts as an entraining 'zeitgeber' (translated as 'time giver') for the internal oscillator (Rensing et al. 2001). An essential property of a circadian rhythm is its persistence in the absence of periodic input into the system. In constant conditions, the period of the autonomous, or 'free-running', rhythm usually deviates from 24 h, but the direction and amount of this deviation depends on species, physiological state, and environmental conditions (Aschoff 1981). For example, in some rodents there is a tendency for the free-running period to be shorter after an advance time shift in light cycle, while others lengthen the free-running period after a delay shift (Pittendrig & Daan 1976), but these changes take more than one cycle (e.g., a cycle of 24 h) to take effect (Nagano et al. 2003). An adaptive benefit of a circadian biological clock is to enable the organism to predict, rather than respond to, daily events

in an organism`s environment (such as the incidence of food, potential mates or predators; Golombek & Rosenstein 2010; Moore et al. 2016), thus facilitating its evolution early on in the history of life (Saunders 2002).

Photoperiodism relates to how animals assess the length of the day as an anticipatory key to time-related events in their life history and thus is affected by both latitudinal and seasonal changes (Bradshaw & Holzapfel 2007). Indeed, daylight length is a crucial parameter in the evocation of facultative diapause in many insects, and as this varies regularly with latitude, insects should vary in their behaviour accordingly at different geographic locations (Masaki 1961). Additionally, long summer days at higher latitudes may affect the onset of activity in animals whose behaviours are under circadian clock control (Pittendrigh 1993; Bradshaw & Holzapfel 2007). Pittendrigh and Takamura (1989) proposed that at latitudes with summer photoperiods exceeding 12 h, the signal underlying the pacemaker's timing function is weakened, and circadian rhythmicity tends to damp out, or even stop entirely, at longer day lengths (Bradshaw et al. 2003). Evidence in support of this is that variation in onset of activity in some mammals and birds is most pronounced at high latitude (Daan & Aschoff 1975). Indeed, the same species in different latitudes may exhibit different reactions to seasonal changes. For example, near the Arctic circle, the sculpin fish *Cottus poecilopus* naturally shifts its behaviour from nocturnal in summer to diurnal in spring, and this phase shift persists when moved South (transfer from 66º to 55º), whereas *C. poecilopus* originally from the South of Sweden maintain a dark-active cycle throughout the year, suggesting that the phase shift in *C. poecilopus* is a characteristic of the high latitude populations (Andreasson 1969; Andreasson & Muller 1969). Nevertheless, while northern *C. poecilopus* maintained their endogenous rhythm when moved from North to South, their onset of activity began distinctly earlier in South of Sweden (Andreasson & Muller 1969). Additionally, bio-ecosystems in high latitudes may be more sensitive to change than those at low latitudes, which may result in a faster response to ambient changes (Root 1989). Above studies suggest that we may expect more sensitivity to change among animals 'moving' from lower to higher latitudes, as could be simulated by a 6 hour advance shift in lights-on versus a 6 h delay shift.

Response to changes in daylight hours may also be due to different clock mechanisms regulating temporal behaviour. Some behavioural and physiological

processes in arthropods (e.g., diapause or reproductive phase in aphids in response to seasonal changes; Lees 1966, 1973) do not appear to adhere to a classic internal oscillator (Saunders et al. 2002). In these cases, the proposed mechanisms underlying timing are referred to as 'hourglass' models. A characteristic of these models is that, without external input (e.g., dawn), the timing mechanism 'dies out' within a cycle, whereas circadian clocks typically require a number of days to reach a complete phase shift (Daan 1987; Bradshaw et al. 2003). A relevant external signal, such as light, can be used to distinguish between circadian clock and hourglass models. If the behaviour is based on an hourglass mechanism, an advance or delay in light regime should produce a quick phase shift, whereas if timing depends on a self-sustaining oscillator, the onset of behaviour should experience a delay relative to the new light regime (Biebach et al. 1991).

Spiders appear to exhibit daily rhythmical facets in behaviour and physiology (e.g., Kovoor et al. 1995, 1999; Yamashita & Nakamura 1999; Nørgaard et al. 2006; Jones et al. 2011), but research investigating activity patterns is still relatively scant in this large taxonomic group (Seyfarth 1980; Schmitt et al. 1990; Suter 1993; Ortega-Escobar 2002; Jones et al. 2011; Moore et al. 2016). With almost 47,000 described species in 113 families, spiders are one of the largest taxa on Earth (World Spider Catalogue 2017). Although many spiders are nocturnal, some or all species within the families Salticidae, Oxyopidae, Thomisidae and Lycosidae are active during the day (Foelix 1996; Cloudsley-Thompson 2000). In particular, salticids (jumping spiders) are active diurnal hunters (Jackson and Pollard 1996; Nelson & Jackson 2011), suggesting a response to daylight changes, yet no published information exists on their circadian features. The Salticidae is the largest family of spiders, comprising over 10% of all spider genera and all spider species (World Spider Catalogue 2017). Their distribution across the globe except the poles makes salticids excellent candidates for investigation on temporal behaviour.

In this study, I investigated short-term temporal effects on salticid behaviour across different light regimes simulating different latitudes. Specifically, I investigated how *M. marina* respond to shortening and lengthening of daylight hours by calculating the level and onset of activity over three successive days in a variety of LD and free-running conditions.

Methods

General Methods

Twenty field-collected (as juveniles) virgin female *Marpissa marina* (Goyen 1892; Araneae: Salticidae) were used for this experiment (*N*= 20). For maintenance, spiders were individually-housed in plastic containers (40 mm diameter × 50 mm) with a ventilation hole covered with mesh and a hole at the bottom containing a cotton wick which hung into a glass of water to provide humidity and drinking water (Jackson & Hallas 1986). An additional hole, plugged with a cork, was used for feeding the spiders with house flies, *Musca domestica*, once per week.

All spiders were fed to satiation on house flies 24 h prior to the experiment (i.e., there were always live prey remaining in the cage after 24 h). For testing, spiders were transferred individually into glass tubes (outer measurements, 16 mm diameter × 100 mm long; TriKinetics, Waltham, MA, USA) to measure locomotory activity. These were enclosed at one end by a snug-fitting small glass tube containing water plugged by a cotton wick that was inserted 10 mm into the locomotory activity tube in order to provide spiders with water throughout the experiment. Locomotory activity tubes were enclosed at the other end by mesh held by a rubber band.

Activity tubes were loaded into specially-designed locomotory activity monitors (LAM) (LAM25, TriKinetics, Waltham, MA, USA) enabling simultaneous recording for up to 32 channels (individual tubes). As each spider moved back and forth in its activity tube, it interrupted one of three infrared light beams that bisected the tube. Each crossing was counted by the system and the activity counts per min for each tube were sent through an interface unit (PSIU9, TriKinetics, Waltham, MA, USA) via USB to a computer. Data were pooled into 30 min bins using the dedicated activity monitoring software (DAM File-Scan, TriKinetics, Waltham, MA, USA) for further analysis.

Monitors were housed inside environmental chambers (Contherm-POLAR1000, 50 cm high X 50 cm wide X 35 cm deep; internal diameters) at a controlled temperature (25^{o}). Light was provided by four strips of 12 wide-beam LED lights purpose-built for the chamber and placed on the inner ceiling to provide

consistent lighting throughout the chamber. Light was set at 10 lux (higher intensities caused arhythmicity under constant light condition in preliminary experiments), as measured in the middle of the chamber where the monitors were placed.

I investigated how shortening and lengthening daylight hours (direction and size of shift hours) affected salticid behaviour, specifically concerning the latency (the delay in onset of activity after as phase shift) and advance onset, and activity levels, defined as the average number of crosses of the infrared beam within 30 min bins. For this, I ran one experiment in sequential five-day parts which the first two days of each cycle (transient phase) were removed from the results (Fig. 1). The series of LD (LD always 12:12) phase shifts began with an LD cycle (LD1) followed by a constant light condition (LL1), followed by an LD cycle with a phase delay (relative to the baseline LD) of 6 h (LD2). This 6 h forward shift was followed by another free-running in LL (LL2), followed by LD with another phase delay (relative to the second LD) of 10 h (LD3). This was followed by two 6 h phase advance and delay, respectively (LD4 & LD5) (Fig. 1).

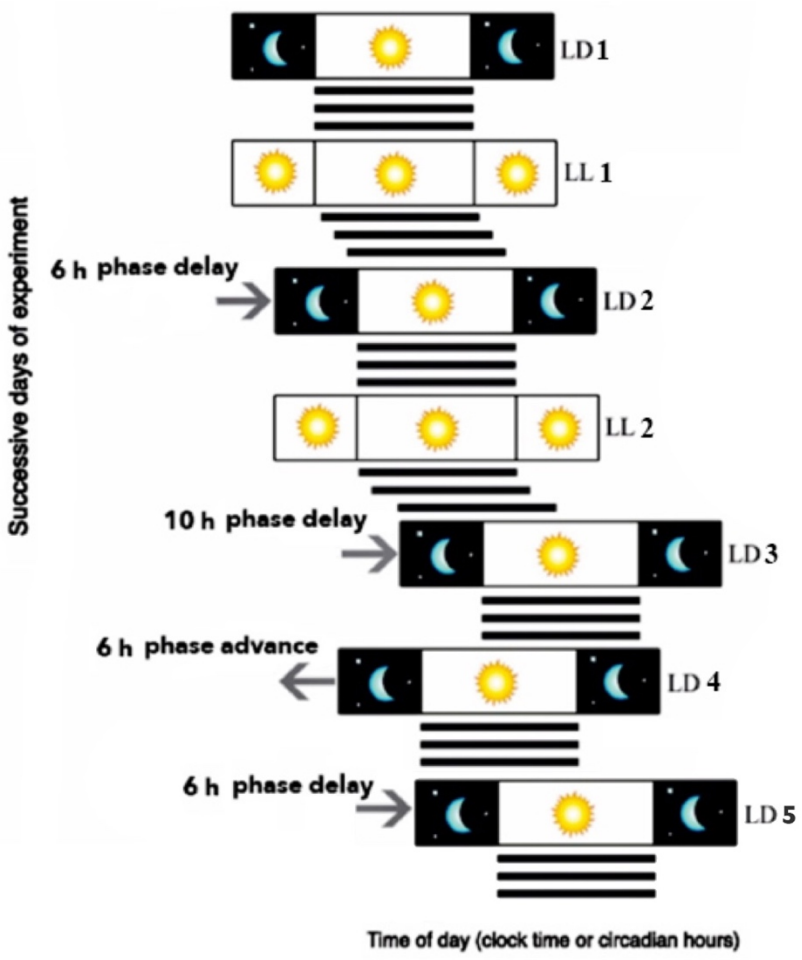

Fig. 1. Abstract representation of experiment; horizontal black bars depict spider activity and vertical axis shows successive days of experiment (modified from Golombek & Rosenstein 2010). LD represents the light/dark cycle and LL shows constant light condition.

To determine the how shortening and lengthening daylight hours affected latency, I considered two days of transient phase based on my preliminary experiments and compared measurements on the first day after that (third day after a phase shift) for LD conditions (as this is when the behaviour is still expected to be under the control of circadian clock and the animal has not yet entrained to the new LD condition), and on day three (after removing two days of transient phase from analysis) in LL conditions (when animals were assumed to be free-running). Specifically, I compared the effect of shift size of light on latency by considering two 6 h and 10 h shift and the effect of zeitgeber direction by advance and delay shifts. I compared the latencies and advance

onsets after the advance and the delay shifts to determine the speed at which spiders adjusted to new light regimes, enabling me to explore the relative effect of hourglass models (immediate adjustment) versus circadian clock models. My hypothesis states that, if the response is driven by circadian clocks, the difference angle (advance or delay in onset of activity) on the third day of a free-running period (when clock effects are evident) with respect to the lights-on time of its previous LD should be the same as the latency on the first day of entrainment after a new advance or delay shift (Fig. 2) (in a clock control behaviour, the LD cycles need a few days to initiate the rhythm and the free-running periods would persist at least for 3-4 cycles (see Cahill 2002 ; Yamazaki et al.2000; Tosini & Menaker 1998). I also compared average activity levels over the three days across all light regimes.

To maintain spider health in this 18-day experiment, in LD2 the test spiders were removed during 'light' and placed in their cages for 4 h and were fed a housefly before being returned, still during 'light', to clean glass tubes in the DAM (in the same channels in which they had been previously). No further feeding of the spiders was done during the rest of the experiment in order to minimise the impact of this on behaviour. I had previously determined that three days was sufficient to observe any changes in activity patterns (after two days of transient phase which is kind of mess up in rhythm of activity).

ImageJ (Rasband 1997-2016) with the ActogramJ plugin was used to plot actograms and one-way repeated measures ANOVA with Bonferroni post-hoc tests were done in Prism. In LD rhythms, latency differences were compared between lights-on and latency to onset of activity (on the first day after transient days) relative to the previous LD. In the free-run cycle, latency on the third day (after transient phase) was measured with respect to the lights-on time in their previous entrainment cycle. To compare the number of spiders exhibiting rhythmic versus arrhythmic behaviour under LL and LD conditions I used Fisher exact tests.

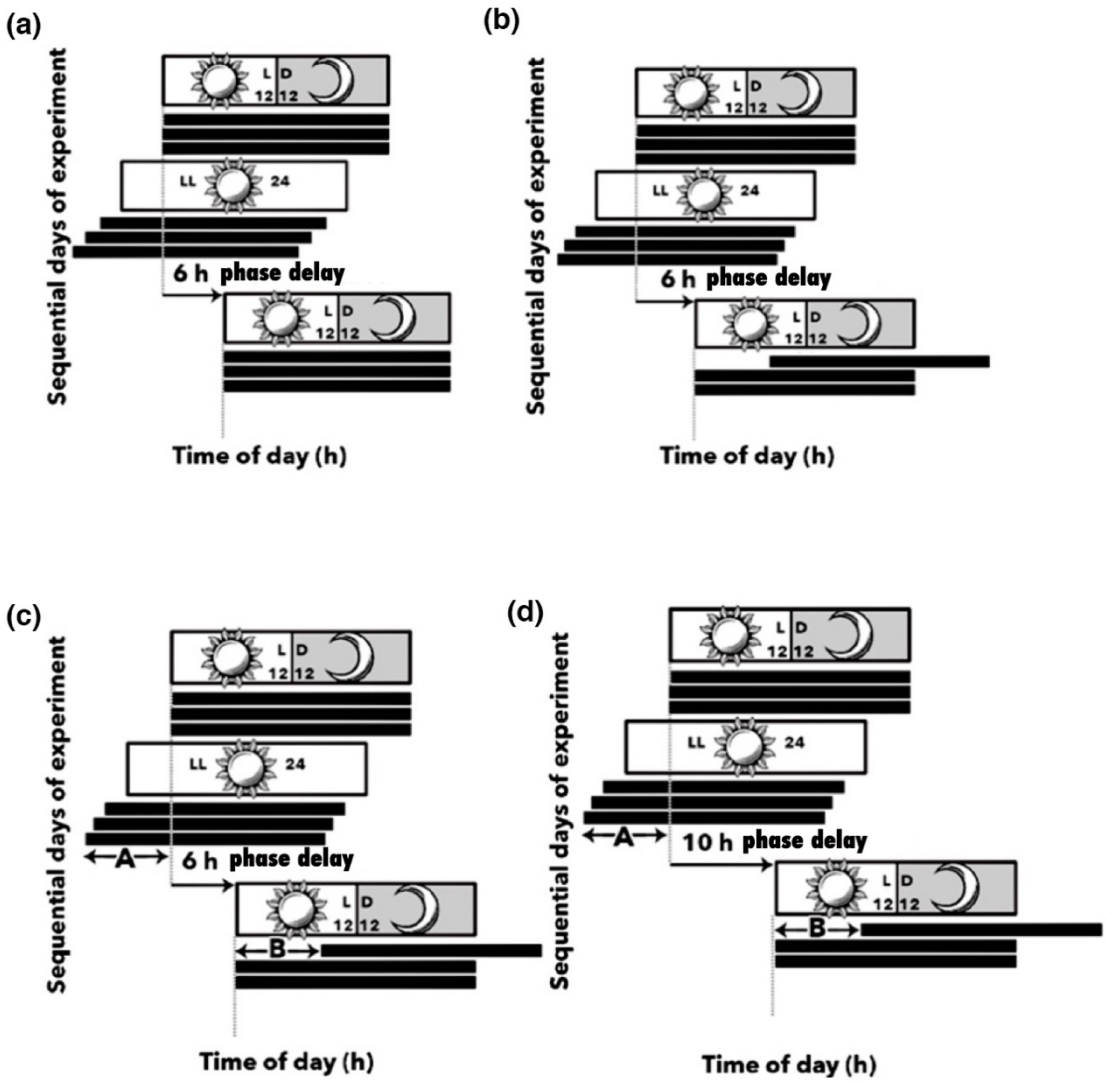

Fig. 2. Representation of predictions under hourglass and circadian clock mechanisms after a free-running period (LL) is changed to an LD situation with 6 and 10 h phase delay from the previous LD condition. Black bars denote activity. **a.** For an hourglass model, I predict an immediate 6 h phase shift in activity. **b.** With a circadian clock I predict the initial phase shift will be delayed > 6 h. With a circadian clock the time difference between lights-on and onset of activity on the first day of a new LD cycle (after removing two days of transient phase) should be equivalent to the advance (or delay) in the onset of activity on the final day of free-running in both 6 h (**c**) and 10 h (**d**) phase delay scenarios (i.e., A=B in both 6 and 10 h phase shifts). LD represents the light/dark cycle and LL shows constant light condition. Horizontal black bars depict spider activity and vertical axis shows successive days of experiment.

Results

Very few spiders became arrhythmic under LL. There was no significant difference between the number of spiders exhibiting rhythmic and arrhythmic behaviour in second LL (rhythmic, n = 17, arrhythmic, n = 3) and its previous LL (rhythmic, n = 18, arrhythmic, n = 2) (Fisher exact test, p = 0.244).

For latency (delay in onset of activity), I was specifically interested in the differences in the onset of activity under different light regimes (LD1, LL1, LD2, LL2, LD3, LD4, LD5). Overall, there was a significant effect of treatment on latency ($F_{6,114}$ = 81.47, P < 0.0001, *N*=20; Fig. 3c). The average free-running period over three days for all spiders in the first LL was 26 h (± 0.84) and in LL2 was 26 h (± 0.81), showing no 'after-effect' of the intervening 6 h delay (LD2) of an LD period (Fig. 3). Besides, in constant conditions (here constant low light regime) the spiders' internal clock (s) tends to be greater than 24 h. In the other words, they showed a latency (delay) in onset of their activity in both LL condition (2 h ± 0.54, *N*=20). Such this latency, was seen just after advance shift by 6 hours (1.8 h ± 0.29, *N*=20) (Fig. 3c**)** which means the mean delay in onset of activity on first day of LD4 (after 6 h advance shift which followed a 10 h delay shift) and third day in all LL treatments (after removing very first two transient phase of each cycle), were the same ($F_{2,38}$= 3.87, P = 0.316, *N*=20; Fig. 3c), but the advance onsets of activity after delay phase shifts differed from latency in LL cycles (apart from size of the shifts). In all LD cycles (except LD 4), spiders advanced their onset of activity (anticipatory behaviour) apart from advance or delay shift. Average activity levels over three days varied with treatment (overall ANOVA, $F_{4,76}$ = 57.45, P < 0.0001, *N*=20; Fig. 3d), and in LD 4 (after advance shift) was significantly higher than in all other treatments (Fig. 3d). Activity levels did not significantly differ after a 6 h delay shift (LD2) compared with a 10 h shift (LD3). The rhythm of activity in the first day of each LD was the same as the third day of its previous LL (endogenous clock control), if followed a constant light condition and the same as the light-on and light -off time in its last LD cycle, if followed an LD cycle directly (exogenous clock control) (Fig. 3b).

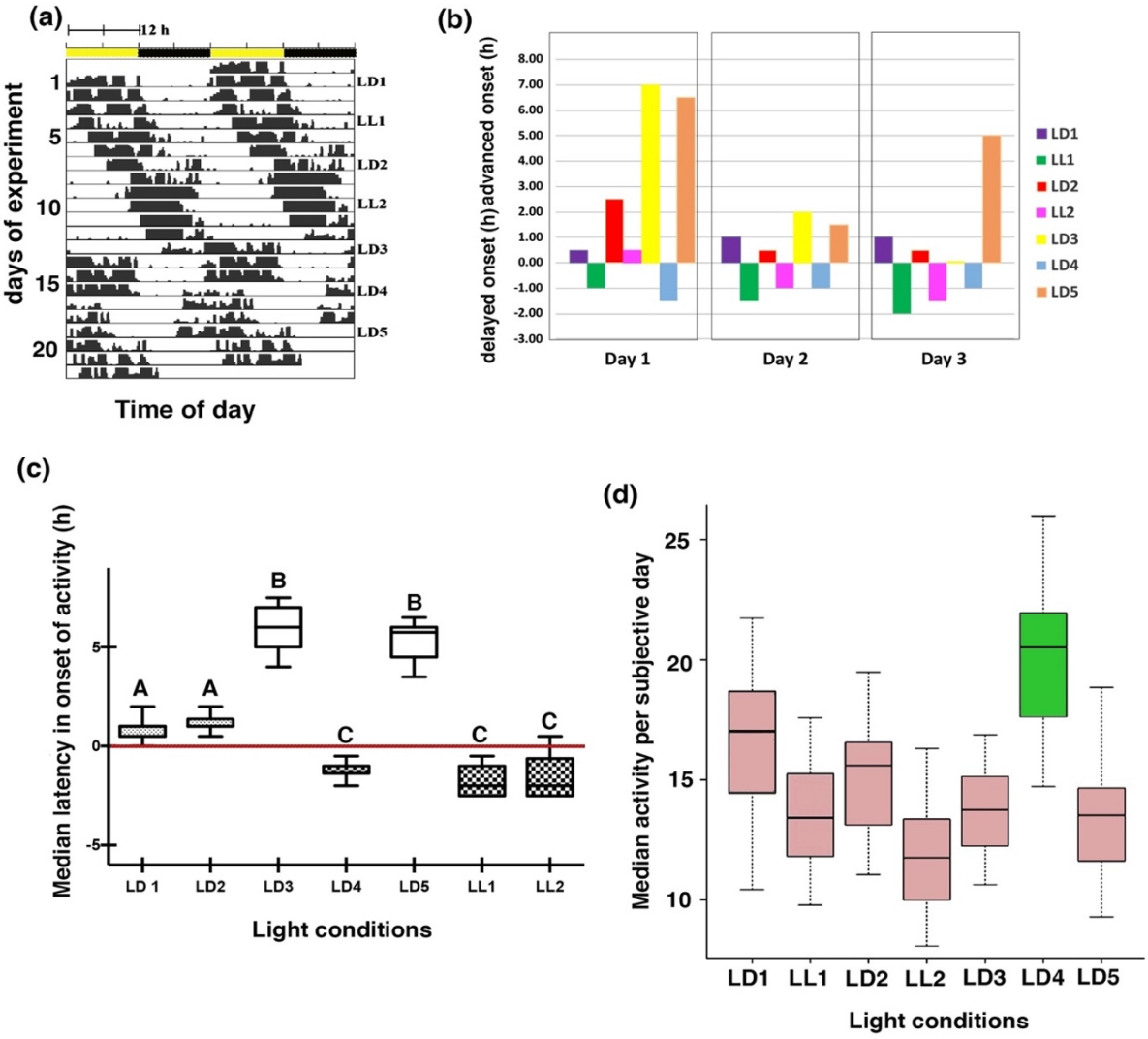

Fig. 3. Representation of *M. marina* (Salticidae) activity (latency and advance in onset and the level of activity) under experiments conditions (LD1, LL1, LD2, LL2, LD3, LD4, LD5). LD represents the light/dark cycle and LL shows constant light condition. **a.** The average 21-days double plotted actogram of activity for all successive parts of experiment. Achtophases are black and the scotophase are white areas. The actogram has been normalized according to the best typical individual (channel 5) and upper and lower limit of activity have been reduced getting rid of extra noises. The very first two days of each cycle considered as transient phase and were removed from the actogram and analysis. **b.** The daily presentation of advance and delay in onset on activity for all experiment parts (first, second and third day in all parts). The lights-on time considered as zero. **c.** Boxplot of latency and advance in onset of activity in all experiment parts. Each box presented median, upper and lower limit of data and SD in each light conditions. The first LL, which happened after normal LD, was considered as a scale for real circadian clock controlled free-running period. All the other conditions happened after light hours shifts. Different letters denote significant differences at alpha = 0.05 (Bonferroni test). **d.** Boxplots of activity median for 12 h day-light (or subjective day for free running periods). Level of activity in LD4 was significantly higher than the other conditions (alpha = 0.05, Bonferroni test).

Discussion

This is the first long-term experiment on spiders in which a series of light-regime phase shifts has been performed. I found that *M. marina* are very resilient to phase shifts up to 10 h, especially if these are in a forward direction (delay shift). It seems a long shift (10 h) is not able to shift the rhythm easily in short period as the spiders reacted to this change with a long delay in onset of their activity. After the advance shifts (shorter days) the spiders showed the same size of latency in onset of activity with those of spiders in constant light condition, suggesting endogenous clock-controlled behaviour. In this case, and under constant lighting conditions, latency to onset of activity was about two hours delayed with respect to initial entrainment. In this condition, when there was a phase advance, activity levels were significantly higher. In all the delay shifts which lengthened nights (or shortened the photoperiod of the first day), and the advance shift, the rhythm was just affected by its last cycle and there was no memory for keeping the initial rhythm or a couple of previous cycles. If the previous cycle was an entrained cycle, the rhythm of activity in first day of new cycle tended to keep that entrainment, and if the last part was a free-running in constant light, the rhythm in the first of the new cycle (after shift) tended to continue the free running, apart from the size and direction of the shift. In this scenario, I found *M. marina* became active sooner on the days after delay shifts (lengthening the nights), in fact anticipating lights-on, but it seems there is a threshold for that because, when the size of shift went up to 10 h (exceeding the threshold(s)) on the third day after shift, spiders were not able to anticipate the light -on any more. This result is contrast with findings that activity in *Drosophila ananassae* populations at Northern latitudes (with longer days) anticipates sunrise, while Southern representatives begin activity after sunrise (Joshi 1999). Regarding the fact that jumping spiders are diurnal, shortening the day may act as a cue for anticipating the bad future conditions. Additionally, activity levels of tadpoles are thought to increase with increasing latitude (Laurila et al. 2008), and my simulated shortened night also elicited significantly higher activity levels in *M. marina*, lending some support to this notion. Differential responses to phase advances and phase delays are well documented. Having made a learned association between time and feeding area, garden warblers showed no effect when subjected to a 6 h phase delay, but

advanced their foraging behaviour by 2.5 h when subjected to a phase advance (Biebach et al. 1991). Also, sugar gliders *(Petaurus breviceps)* re-entrained with less latency after 8 h delay shifts of LD cycle than after 8 h advance shifts (Kleinknecht & Erkert 1991). In mammals, the mechanisms underlying these appear to rely on melanopsin in retinal ganglion cells (Panda et al. 2002; Ruby et al. 2002), but little is known about this process in invertebrates, which have a dramatically different set of visual processing systems – and eyes – to those of mammals (Land & Nilsson 2012). About the spiders it seems they need less time to re-entrain to the new cycle after advance shift. Sparrows (Eskin 1971), Japanese quail (Boissine et al. 1976), mice and hamsters (Pittendrigh & Daan 1976) and slime moulds (Schemidlei 1951) exhibit a longer free-running period when moved from longer light photoperiod conditions to constant conditions than when moved from shorter photoperiods. In practice, this means that moving from longer days to shorter days induces gradually shorter free-running as an aftereffect of the photoperiod, but no after effect was seen in free-running of jumping spiders. In this study, on first day of several of the advance phase shifts, I simulated an increase in day length and expected an increase in the free-running period after a 6 h advance shift, but found no such aftereffect. This result may be because the apparent length of the photoperiod was only altered on the first day of the cycle (which remained at 12:12), or because three days in constant conditions was insufficient to get an accurate portrayal of the free-running period, which might have still been somewhat entrained or may not be effective in free-running of these spiders.

It has been suggested that animals experiencing long photoperiods (e.g., at high latitudes) will have damped circadian rhythmicity, and must respond faster to photoperiod conditions for survival in terms of growth and development at high latitudes with very short summers (Wegis et al. 1997; Merilä et al. 2000; Angilleta & Michael 2001; Bradshaw et al. 2003; Laugen et al. 2003; Uller & Olsson 2003; Lindgren & Laurila 2005). As such, these animals may rely on hourglass mechanisms. Controversially, Lees (1965) showed lengthening dark hours acts like a stimulus for hourglass timing system in the vetch aphid, *Megoura viciae,* where the response was quick and led to reproductive mode. In *M. marina*, I found significantly faster reaction in onset of activity after an abbreviated night (phase advance). However, in no case did I observe an immediate phase-shift in keeping with the new light regime, as would be

expected with hourglass models. Also, it was notable that inducing a very long night (10 h delay shift) after free running and lengthening the night by moving from high to low latitudes caused a remarkable response in onset of spider`s activity. These results provide no support that *M. marina* spiders adhere to hourglass model of timekeeping, and instead rely on biological (circadian) clocks models with a period of close to 24 h to maintain a thoroughly resilient diurnal pattern of behaviour.

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Chapter 5

Investigation of masking effects of nocturnal and diurnal light intensities on locomotor activity of jumping spiders

Abstract

Besides permitting vision and orientation in space, light elicits several acute physiological effects like increasing core body temperature and heart rate, modulating activity level and affecting the circadian clock. In nocturnal animals, light usually inhibits activity, whereas it promotes activity in diurnal animals. Continuous dim light strongly affects the period and the power of the free-running rhythm, and continuous light exceeding a certain threshold may provoke arrhythmicity. When direct effects of light are present, they often conceal the actual entrained circadian activity rhythm; for this reason, they are called "masking effects". The aim of this study is to determine the mechanism by which this temporal phasing is controlled in *Marpissa marina* (Goyen 1892; Araneae: Salticidae). For this, I examine spider`s locomotor activity and anticipatory behaviour which may be influenced by total darkness (new moon), nocturnal dim light (full moonlight emulating), and intense (bright) light and 'normal' light. My results suggest the ability of *M. marina* to anticipate the start and end of daylight hours is not related to light intensity during the scotophase (dark cycle), but is related to illumination during daylight hours. Diurnal illumination does not affect salticid activity, while nocturnal illumination significantly increased activity compared to total darkness, which appeared to have a masking effect on salticid activity. A clear masking effect on *M. marina*'s clock was found under bright light, in which condition *M. marina* were no longer able to anticipate lights-on and lights-off. In addition, the free-running period of spiders under constant normal light reduced to < 24 h, while under moonlight intensities, increased to > 24 h.

Introduction

Endogenous circadian clocks prepare organisms according to the reliable and predictable changes in the cycle of day and night. To function as reliable timers, circadian clocks are synchronized to the 24-h cycle, which is accomplished mainly by

light (Bachleitner et al. 2007). Light influences animals in many ways. Besides permitting vision and orientation in space, it elicits several acute physiological effects. Light increases core body temperature and heart rate (Cajochen et al. 2005; Erkert & Gröber 1986; Gander & Moore-Ede 1983), and it strongly modulates activity level (Aschoff & von Goetz 1989; Binkley & Mosher 1985; Erkert & Gröber 1986). In nocturnal animals, light usually inhibits activity, whereas it promotes activity in diurnal animals (reviewed by Mrosovsky et al. 1999). In addition to these effects of light on activity and physiology, light also strongly affects the circadian clock. Light/dark cycles are the most important zeitgebers that entrain circadian clocks to the 24 h cycles of the environment. The important point here is that bright light during the day has little effect on circadian clocks. In nature, stable synchronization is a challenging task because, irradiances during day can vary largely from day to day because of the weather. Bünning in 1969 measured irradiances systematically throughout day and night and found that day-to-day fluctuations are smallest before sunrise and after sunset and late dusk, when the irradiances are under 10 lux. Therefore, Bünning (1969) proposed that organisms time their clocks to very low irradiances and thus must be very light-sensitive, as he found that bean plants synchronize to light of moonlight intensity (0.6–0.8 lux).

In animals, it is under debate whether natural, dim, nocturnal light affects the endogenous clock, and indeed, most studies in mammals neglect the possibility that the clock may be highly light-sensitive. For instance, activity was recorded under nocturnal dim light ranging from starlight to full-moonlight intensity, assuming that dim light has little influence on the circadian pacemaker. However, it has been shown that nocturnal light can affect activity levels as well as activity patterns (Erkert & Cramer 2006; Erkert & Gröber 1986; Kappeler & Erkert 2003; Erkert 1975; Fernandez-Duque & Erkert 2006). Continuous dim light strongly affects the period and the power of the free-running rhythm, and continuous light exceeding a certain threshold may provoke arrhythmicity (Aschoff 1979; Konopka et al. 1989). The direct effects of light on physiology and the light effects exerted through the circadian clock on activity are sometimes hard to distinguish. When direct effects of light are present, they often conceal the actual entrained circadian activity rhythm; for this reason, they are called "masking effects" (Aschoff et al. 1982; reviewed by Mrosovsky 1999).

In some organisms, the amount of moonlight influences nocturnal activity as well as diurnal activity (Fernandez-Duque& Erkert 2006). The term 'cathemeral' was introduced by Tattersall (1987) to describe an activity pattern found in several lemur species where animals may be active both during day and night (Engqvist & Richard 1991). Evolution of this activity pattern among primates revived the open question of how diurnal behaviour may have evolved in an originally nocturnal species (Van Schaik & Kappeler 1997; Kappeler & Erkert 2003) and opens a research avenue to investigate masking effects. For example, cathemeral primates appear to have strong preferences for certain light conditions; they avoid being active under both total darkness, because it precludes visual orientation, and avoid high irradiances, because it may damage their sensitive eyes. This means that total darkness and intense light inhibit activity on these primate's activity. In turn, these results suggest that the variation of nocturnal illumination may shift the clock. The circadian clocks of many organisms are very light sensitive, as shown for the lunar clock of the intertidal midge *Clunio marinus* (Fleissner et al. 2008) and in the fruit fly *Drosophila melanogaster* (Bachleitner et al. 2007; Helfrich- Förster et al. 2002; Rieger et al. 2007). Bachleitner et al. (2007) showed that dim light has the capability to phase-shift the clock. On the other hand, artificial moonlight strongly increased the nocturnal activity level of *D. melanogaster*; most flies converted their activity from diurnal to nocturnal (Bachleitner et al. 2007).

Different properties of light may affect the clock in different ways. For instance, in *Drosophilla,* dim constant light lengthens the free-running period, but intense light causes an arrhythmicity (Yoshii et al. 2005). At the appropriate intensity of light, animals even can anticipate and respond appropriately to regular events (e.g., in rabbits, Jilge 1995, and in *Drosophila*, Wheeler et al. 1993), while strong illumination or total darkness might destroy the clock function, leading to arhythmicity or a masking of activity (Helfrich-Förster 1998). For example, owl monkeys show luminance-dependent activity at approximately 0.1– 0.5 lux (which corresponds to the brightness of the night sky at full moon) (Fernandez-Duque & Erkert 2006). Erkert and Gröber (1986) showed changes in owl monkey activity may be induced by the new moon (essentially darkness), and the waxing and waning of the moon. Similarly, white-fronted lemurs in laboratory conditions switched their activity from primarily nocturnal to diurnal after nocturnal illumination was reduced below a certain threshold (Erkert &

Cramer 2006). This switching appears to be due to masking effects, because activity always started to free-run after a preceding dark phase rather than a light phase after the animals were transferred from light/dark conditions to constant darkness (Erkert & Cramer 2006). This means that the endogenous clock of lemurs was not shifted during the switched activity pattern: the animals remained principally nocturnal, even if they showed activity only during the day (Erkert & Cramer 2006).

Spiders rely on their circadian clocks to regulate behaviours and physiological processes (Seyfarth 1980; Schmitt et al. 1990; Suter 1993; Yamashita & Nakamura 1999; Ortega-Escobar 2002; Jones et al. 2011). It is widely believed that most spiders are nocturnal, avoiding diurnal predators such as wasps and birds (Cloudsley-Thompson 1958), yet some spiders are diurnal, while other species forage around the clock (Moore et al. 20016). While nocturnal species may benefit from reduced predation from visual predators, there may be costs, such as reduction in prey density at night, and, at higher latitudes, a shortened foraging period in summer, when these spiders tend to be active (Wise 1993).

Evidence from some nocturnal spiders under laboratory (Ramousse & Davis 1976) and natural (Ceballos et al. 2005) conditions, as well as from diurnal spiders during a total solar eclipse (Uetz et al. 1994) suggests that some activity in spiders may respond directly to light conditions, rather than being controlled by a light-entrahinable endogenous circadian rhythm. If the phasing of a behaviour is under circadian clock control, then, under constant conditions, the behavioural rhythm should continue (free-run) with the period of internal clock. Alternatively, if a behaviour is controlled by exogenous factors (i.e., it responds directly to changes in light levels), then that behaviour should occur immediately (or shortly) after dusk or dawn, and the rhythm should not persist under constant conditions.

Although many spiders are nocturnal, species within the families Salticidae, Oxyopidae, Thomisidae and Lycosidae are active during the day (Foelix 1996; Cloudsley-Thompson 2000). In particular, salticids (jumping spiders) are active diurnal hunters (Jackson and Pollard 1996; Nelson & Jackson 2011), suggesting a response to daylight changes, yet no published information exists on their circadian features. With more than 46,000 species in c. 4,000 genera in c. 120 families, spiders are one of the largest taxa on Earth. The salticids alone comprise over 10% of all spider genera and

all spider species (Platnick & Raven 2013).

The primary objective of the present study is to describe the exact timing of locomotor activity in the salticid *Marpissa marina* (Goyen 1892) with respect to light:dark cycles and then to determine the mechanism by which this temporal phasing is controlled. For this, I tested the effect of total darkness (new moon), nocturnal dim light (full moonlight emulating), and intense (bright) light and 'normal' light on their activity. I exposed individuals to conventional laboratory 'normal' light:dark (L_nD) cycles and subsequently to constant total darkness (DD) to determine the activity pattern of spiders and observe any free-running patterns of activity. With a normal light: 'moonlight' (L_nM) cycle (nocturnal dim light as moonlight intensity) following a constant moonlight nocturnal night (MM), I compared the pattern of activity with previous experiment to investigate the existence of masking effects in total darkness and revealing the real activity pattern of jumping spiders in nature (in full moonlight). I continued the experiment with intense (bright) light:dark cycles (L_iD) following constant normal light L_nL_n and intense light L_iL_i to investigate masking effects in intense light conditions.

Methods

General Methods

Twenty field-collected (as juveniles) virgin adult female *Marpissa marina* (Araneae: Salticidae) were used for this experiment. Between each part of the experiment, spider had one week rest under normal LD 12:12. Daylight intensity in the micro habitat (under rocks and coastal algal debris) of *M. marina* was measured as 100 lux by means of light-meter (LI.COR, model LI-250), which was used as the 'normal' intensity in my experiments. The maximum brightness in a sunny day in their habitat was measured at 300 to 400 lux, and as such I considered 300 lux as intense (bright) illumination. Illumination in their habitat at full moonlight was measured at 0.5 to 0.8 lux and I used 0.8 lux as nocturnal light (full moonlight).

For maintenance, spiders were individually-housed in plastic containers (40 mm diameter × 50 mm high) with a ventilation hole covered with mesh and a hole at the bottom containing a cotton wick which hung into a glass of water to provide humidity and drinking water (Jackson & Hallas 1986). An additional hole, plugged with a cork, was used for feeding the spiders with house flies, *Musca domestica*, once per week. The animals were released following experiments. All spiders were fed to satiation on house flies 24 h prior to the experiment (i.e., there were always live prey remaining in the cage after 24 h).

For measuring locomotory activity all methods were as described in Chapter 2. Here, I ran three experiments consisting of a five-day LD cycle (LD always 12:12), followed by constant conditions. At least one week separated each experiment. The first experiment (Fig. 1a) consisted of a L_iD cycle with intense light at 300 lux followed by seven-days at constant intense light (L_iL_i). The second experiment (Fig. 1b) consisted of an L_nD cycle with normal light, at 100 lux, followed by seven-days at normal light (L_nL_n). The third experiment (Fig. 1c) began with five-days under normal light and full moonlight at 0.8 lux, or L_nM. This was followed by seven-days under a constant full moonlight condition and then another seven-days under total darkness. Light within the chamber was measured in the middle of the chamber where the monitors were placed.

To maintain spider health in this experiment, prior to the constant periods in spiders were removed during 'light' and placed in their cages for 4 h and were fed a housefly before being returned, still during 'light', to clean glass tubes and replaced in the LAM. No further feeding of the spiders was done in rest of the experiment in order to minimise the impact of this on their behaviour. All spiders were returned to the channels within the LAM in which they had been previously placed. I have previously determined that three days is sufficient to observe changes in activity patterns so I considered five days for light:dark cycles and seven days for constant periods to be ample to determine the effects of light intensity on behaviour.

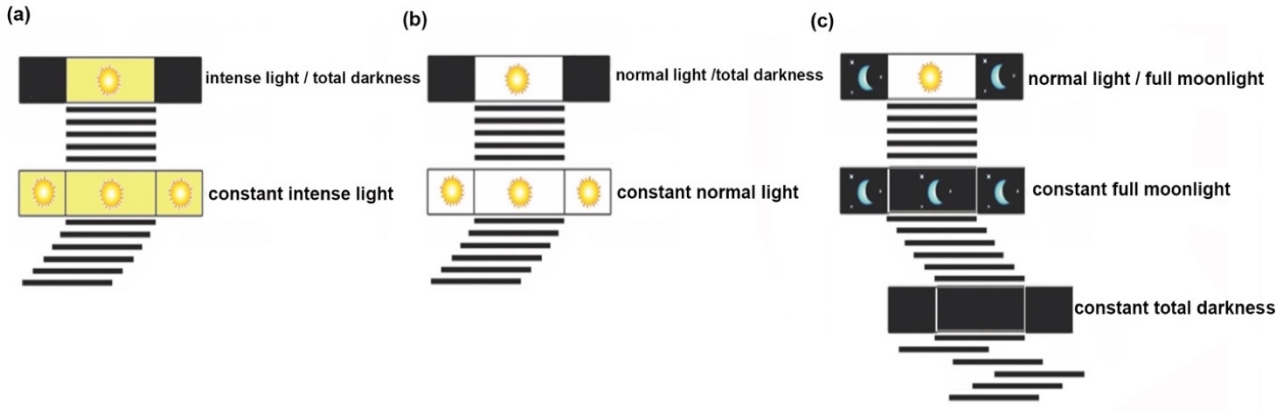

Fig. 1. Experimental protocol for determining the effect of light intensity on salticid activity. For each of the three experiments a 5-day LD (12:12) cycle was followed by constant conditions. **a.** LD at intense-light:total dark, followed by seven days under constant intense light. **b.** LD at normal-light:total dark, followed by six days under constant normal light. **c.** LD with normal-light:full-moonlight, followed by six days under constant full-moonlight and another six days under constant darkness. Horizontal black bars depict spider activity and vertical axis depict successive days of experiment.

Data analysis

I analysed the start and end time of activity under L_iD, L_nD and L_nM entrainment cycles. I also analysed the mean activity levels in light hours and dark hours separately, as well as activity levels in all constant conditions, where L_iL_i, L_nL_n, MM and DD were measured and compared to each other. ImageJ (Rasband 1997-2016) with the ActogramJ plugin was used to plot actograms. In rhythmic freerunning periods, manual phase-angle difference tool was used to measure the differential time from 24 h. Activity was depicted graphically using ImageJ by foursome-plotted actograms to facilitate visual recognition of periods. Periods were detected using two different periodogram analyses, chi-square and Lomb-Scargle. The chi-square periodogram (Sokolove & Bushell 1978) is applicable for analysing circadian data, while the Lomb-Scargle periodogram, using a type of Fourier spectral analysis, is better suited for analysing records with large or frequent gaps (Van Dongen et al. 1999). Using these

two complementary methods, rather than just one, provides additional support for determination of period, as the free-running periods typically need at least 8 cycles (with fewer cycles, the computed value will still be accurate, but the significance test will be less sensitive; Sokolove & Bushell 1978), but because of survival limitations I only had six cycles. Spider survival was problematic because it was not possible for me to mimic full moonlight out of the incubator to feed spiders. In the first day of total darkness, I fed the spiders under red light and I remover the data from the 2 first days of darkness. In rhythmic free-running periods, after recognising the rhythm using a chi-square periodogram, a Lomb-Scargle periodogram was used to find the peak. These statistics were compared to measurements obtained using the manual phase-angle difference tool in ImageJ to check for accuracy. The level and onset and offset of activity data collected from different light conditions were further analysed and graphed in Prism and Excel using one-way repeated measures ANOVA and Bonferroni post-hoc comparisons. Onset and offset of activity and length of the active phase through the day was coded into R (Rjags, Dplyr, Ggplot and Ggvis packages).

Results

All results are reported as the mean number of interruptions of the IR lights, indicating mid-tube crossings ± SD in each 30 min time bin for activity levels over 5 days in light/dark cycles and over 6 days in constant conditions. The mean latency/advance in onset/offset of activity is also used.

The start time of activity under L_iD cycle showed that 85% of spiders started their activity within the first 30 min after lights-on in two first days and started their activity with a latency less than one hour (-0.22 h ± 0.08 , *N*=20) in the following three days (Fig. 2a). Under L_nD cycle, the spiders started their activity immediately on the first day, but from day 2 they started their activity one to two hours before lights-on time (1.04 h ± 0.48 , *N*=20) (Fig. 2a). Under L_nM, once again spiders became active immediately upon lights-on in day one, but anticipated the lights-on time by 2 to 3 h in the last four days in this condition (2.20 h ± 0.43, *N*=20) (Fig. 2a). Also, there was the

significant difference between lights-on anticipation (advance in onset of activity) under all the treatments (L_iD, L_nD and L_nM) ($F_{2,38}$ =164.40, P < 0.0001, *N*=20; Fig. 2b).

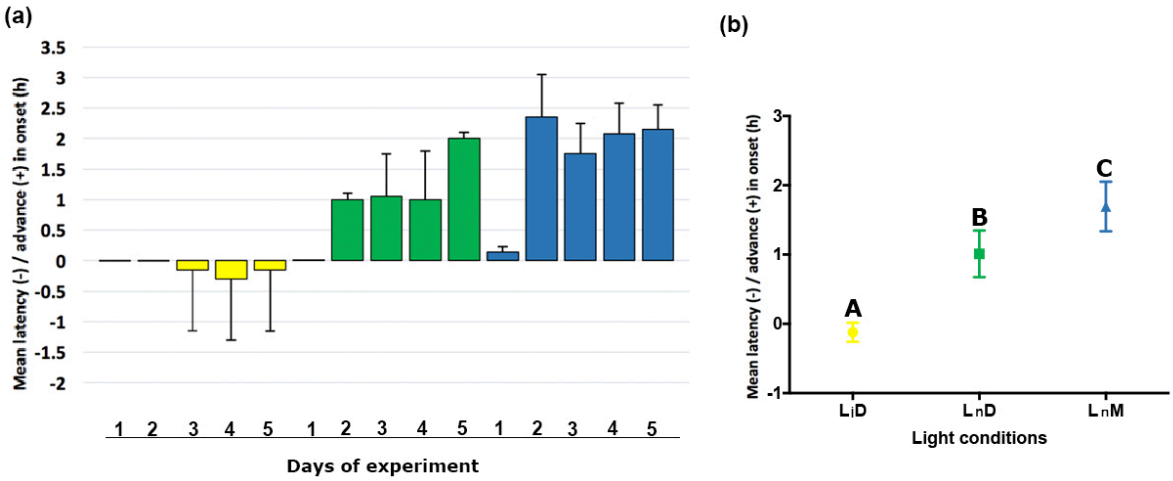

Fig. 2. Representation of onset of activity over five days under L_iD (yellow) in M. *marina*, L_nD (green) and L_nM (blue) entrained cycles. Note: zero represents lights on. **a.** Daily mean activity. **b.** Overall mean in onset of activity. Letters denote significant differences (alpha = 0.05, Bonferroni test). L_iD represents the intense light/dark cycles, L_nD depicts the normal light/dark cycles and L_nM shows the normal light/moonlight cycles. There was the significant difference between lights-on anticipation (advance/delay in onset of activity) under all the treatments (L_iD, L_nD and L_nM). Error bars are SEM, *N*=20.

Spiders in the L_iD condition ended their activity 1-2 h after lights-off (-1.26 h ± 0.36, *N*=20), but in the L_nD cycle, the spiders anticipated lights-off by 0.5 to 1.5 h (1.15 h ± 0.44, *N*=20), as they did in the L_nM cycle (0.94 h ± 0.47, *N*=20) (Fig. 3a). Spiders under L_iD showed significantly less anticipatory behaviour towards the light-off time than the other light conditions (L_nD & L_nM) ($F_{2,38}$ = 168.6, P < 0.0001, *N*=20; Fig. 3), and under both L_nD and L_nM conditions, spiders similarly anticipated lights–off (Fig. 3b).

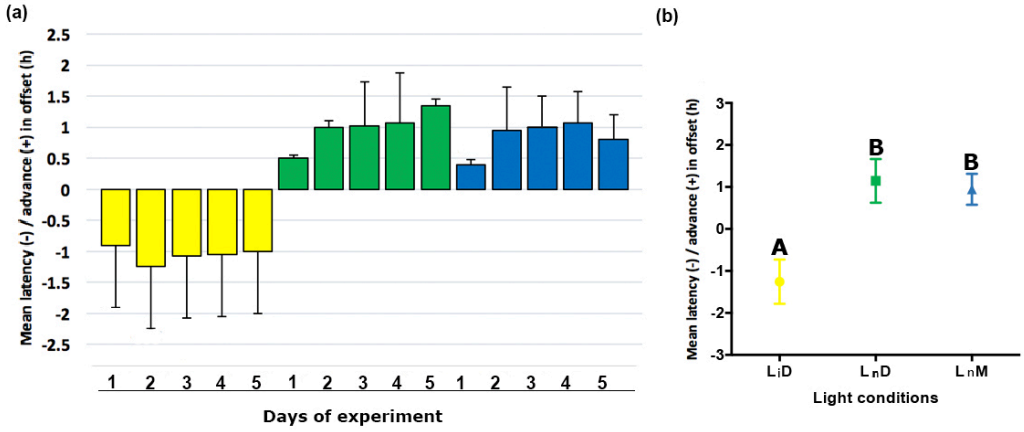

Fig. 3. Representation of cessation (offset) of activity over five days under L_iD (yellow), L_nD (green) and L_nM (blue) entrained cycles for *M. marina*. Note: zero represents lights on. **a.** Daily mean activity. **b.** Overall mean in cessation of activity. Letters denote significant differences (alpha = 0.05, Bonferroni test). L_iD represents the intense light/dark cycles, L_nD depicts the normal light/dark cycles and L_nM shows the normal light/moonlight cycles. Spiders under L_iD showed significantly less anticipatory behaviour towards the light-off time than the other light conditions (L_nD & L_nM). Error bars are SEM, *N*=20.

Mean activity under the L_iD condition during light hours was 22.95 ± 6.45 per day, while during darkness it was 1.99 ± 0.34. Activity in light hours in the L_nD condition was 21.27 ± 6.95 and in dark hours was 2.01 ± 0.91. Under the L_nM conditions, activity was 21.65 ± 7.57 in light and 4.18 ± 0.18 in moonlight. There were significant differences between treatments ($F_{5,95} = 316.3$, $P < 0.05$, *N*=20), with spiders showing significantly higher activity under intense light and showing their lowest activity level under total darkness (new moon condition). Activity under the moonlight condition was significantly higher than under total darkness and significantly less than activity in any other light condition (L_i & L_n).

Mean activity across successive six days (in subjective days and nights, i.e., the times which were supposed to be light/dark in last LD cycle) under constant conditions (L_iL_i, L_nL_n, MM and DD) were: 4.46 ± 1.34 , *N*=20 under L_iL_i, 5.33 ± 1.8, *N*=20in L_nL_n, 5.03 ± 1.3, *N*=20 in MM, and 1.37 ± 0.20, *N*=20 in DD. The spiders were significantly less active under the constant intense light condition compared with normal lighting

and moonlight conditions ($F_{3,57} = 164.13$, $P < 0.05$, N=20). In constant total darkness, activity was minimal, and significantly lower than in all other conditions.

Under constant intense light (L_iL_i) the mean line (the best representative of the mean period for most of the fluctuations over 24 h) of activity over six days was about 5, with high fluctuations around the line (Fig. 4). The line of mean activity under the L_nL_n condition was similar, but had less fluctuations around the line (Fig. 4). The Chi-square periodogram for six days under all the constant conditions (L_iL_i, L_nL_n, MM and DD) showed a clear rhythm of free-running only in the L_nL_n and MM conditions. The Lomb-Scargle periodogram for the L_nL_n and MM showed a strong rhythmicity with PN statistic of 10 (Figs. 4, 5). This periodogram for the periods under L_nL_n showed a significant peak 22 h ±0.6 after lights-on time in its last entrained cycle (L_nD). The Lomb-Scargle table provided 1.5 h value difference in onset of activity, which is similar to that measured manually with the triangle difference angle tool in ImageJ, such that it can be concluded that after six days under constant normal light L_nL_n, the period of free-running (controlled by endogenous clock) is < 24 h.

With a QP (a factor that evaluates the strength of rhythms; Refinetti 1993, 2004) value of 60, the rhythm was not significant for both L_iL_i and DD periods and spiders showed some arrhythmicity in these conditions (Fig. 4, 5). The same measurement in the Lomb-Scargle periodogram in constant full moonlight (MM) condition depicted a significant peak at 25 h±0.45 after lights-on time in its last entrained cycle (L_nM). This corresponded to 2 h in Lomb-Scargle table and ~1.48 h as measured manually (PN statistic; Fig. 5) and it can be interpreted that the free-running period after six days under full moonlight condition delayed from 24 h by ~ 1.5 h.

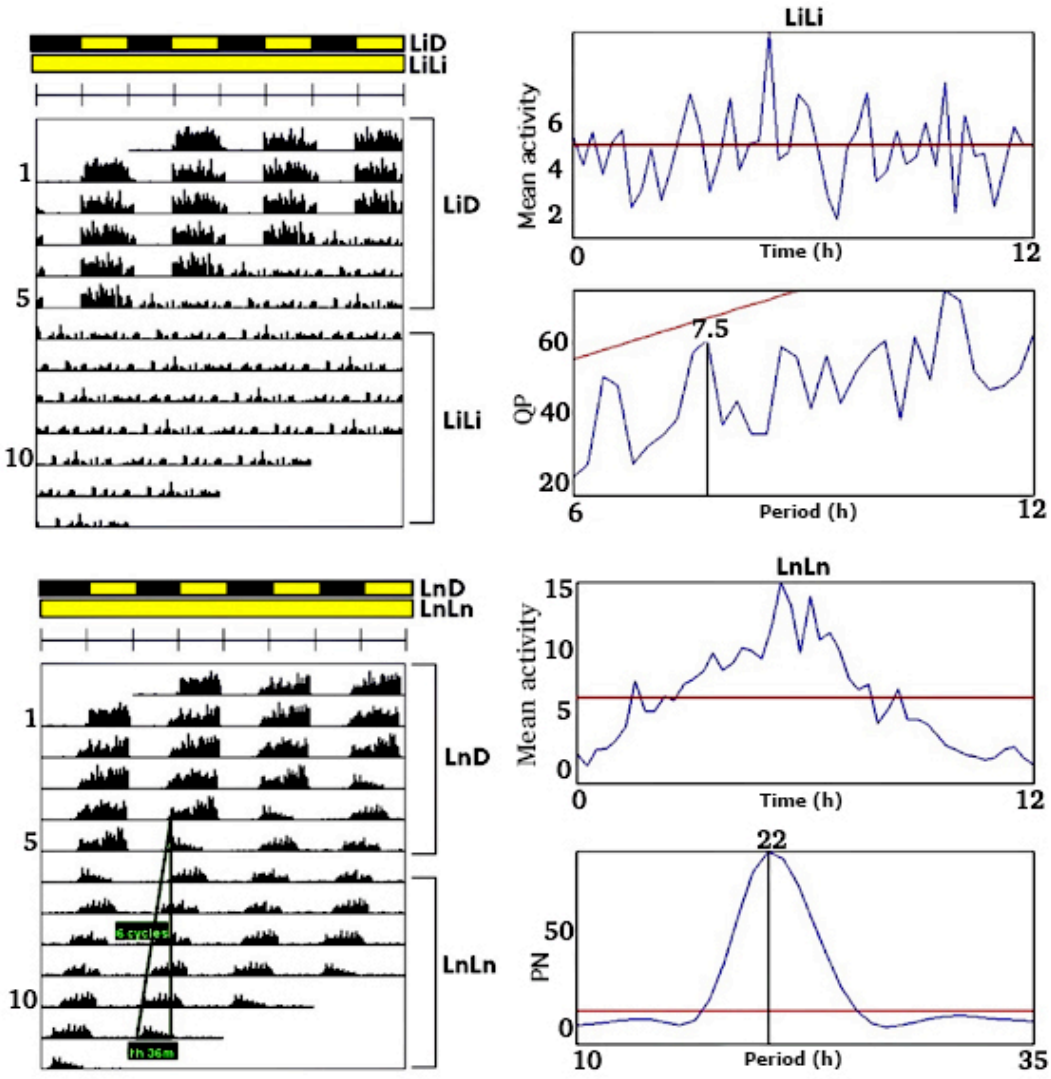

Fig. 4. Actograms (left), diagram of average activity (averaged from all individual periodograms of all spiders) and periodogram (right) of activity of *M. marina* (Salticidae) under constant conditions (L_iL_i, L_nL_n). Actograms of entrained cycles show a tidy rhythm of 12:12. The horizontal lines of actograms depict the days of experiments and the histograms depict activity. The lighting pattern for each condition is shown above each actogram. The Chi-Square periodogram was used to recognize the rhythm in constant conditions and in rhythmic free-running periods, Lomb-Scargle periodogram was used to find the significant peak. This value was checked later in Lomb-Scargle table to find the advance or delay time deviating from 24 h. QP in Chi-Square periodogram and PN in Lomb-Scargle periodogram represent the value of these periodograms for the dataset and the red lines show the level of significance in both periodograms. The triangle difference angle tool in ImageJ was used as a manual alternative to find the advance or delay in free-running periods with respect to their last entrained cycles. In constant conditions in which spiders showed arrhythmicity, the Chi-Square periodogram power lines do not cross any of peaks, showing no significant rhythm in activity patterns. L_iD represents the intense light/dark cycles, L_nD depicts the normal light/dark cycles and L_nM shows the normal light/moonlight cycles. L_nL_n is the constant normal light condition and L_iL_i constant intense light condition.

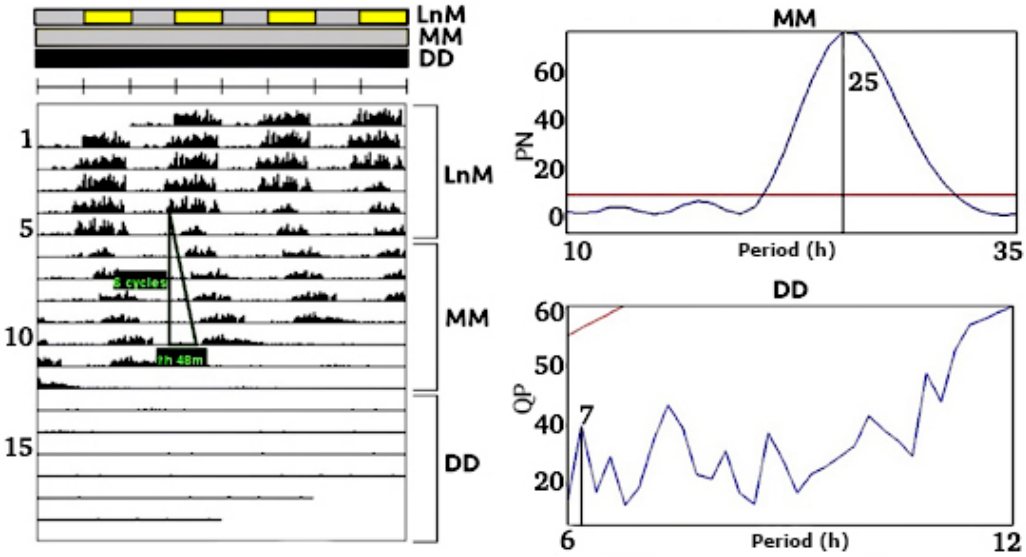

Fig. 5. Actograms (left), diagram of average activity **(**averaged from all individual periodograms of all spiders) and periodograms (right) of activity of *M. marina* (Salticidae) under constant conditions (MM and DD). The horizontal lines of actograms depict the days of experiments and the histograms depict activity. The lighting pattern for each condition is shown above each actogram. The Chi-Square periodogram was used to recognize the rhythm in constant conditions and in rhythmic free-running periods, Lomb-Scargle periodogram was used to find the significant peak. This value was checked later in Lomb-Scargle table to find the advance or delay time deviating from 24 h. QP in Chi-Square periodogram and PN in Lomb-Scargle periodogram represent the value of these periodograms for data set and the red lines show the level of significance in both periodograms. The triangle difference angle tool in ImageJ was used as a manual alternative to find the advance or delay in free-running periods with respect to their last entrained cycles. L_nM is the cycle of normal light/moonlight condition and MM is constant moonlight. DD shows total darkness.

Discussion

My results suggest the ability of *Marpissa marina* to anticipate the start and end of daylight hours is not related to light intensity during the scotophase (dark cycle), but is related to illumination during daylight hours. At normal light intensity (diurnal illumination) coupled with either a total darkness cycle or with a moonlight cycle (nocturnal illumination), salticids can rely in their circadian clock to anticipate the start and end of the day by one to two hours, showing clear diurnal patterns. While little is

known about the genes underlying the clocks of arachnids, in the well-studied arthropod *Drosophilla*, it is the nocturnal illumination which evokes an advance of the morning activity (Bachleitner 2007). It seems that anticipatory behaviour under clock control should be seen in organisms at physiologically tolerable light intensities (that do not cause masking effects on behaviour). For instance, under 500 lux, considered as the normal light intensity for *Drosophila*, insects showed a robust anticipatory behaviour with respect to lights-on (Zhang et al. 2010), which has been demonstrated to be a property of circadian clock through the action of the *period* gene (Saunders & Bertossa 2011; Vanin et al. 2012), while total darkness (but not moonlight) has a masking effect on *Drosophila* activity (Bachleitner 2007).

There was no significant difference between the level of activity under normal and bright light, suggesting that diurnal illumination does not affect salticid activity, while nocturnal illumination does. Moonlight illumination significantly increased activity compared to total darkness, which appeared to have a masking effect on salticid activity. Cerveira (2007) showed that *Cyrba algerina* (Salticidae) can detect and catch prey in very dim light, and she suggested that living in a dim light microhabitat has favored the sensitivity of spatial acuity in *C. algerina*. As at least some salticids evidently can be active in low light conditions, the question then arises of whether and how the animals might regulate their activity on cloudy nights. Williams (1936) demonstrated that noctuid moths are sensitive to moonlight even on cloudy nights, and believed that full-moon nights are warmer than no-moon periods and that the interaction between the temperature and light is synergistic. Here, I saw major differences in nocturnal activity depending on light, but temperature was kept constant at 20˚C. That there may be an interaction between temperature and activity at night in salticids is suggested by my findings in Chapter 2, which show that salticids are somewhat sensitive to temperature in addition to daylight hours, reducing activity at cold temperatures, irrespective of the number of daylight hours.

While salticid activity levels under constant light were similar for all treatments containing some light (including moonlight), the actograms showed that under constant intense light, the locomotor activity of spiders showed some arrhythmicity, suggesting a breakdown of the mechanisms underlying the endogenous clock, perhaps through the action of intense light. A clear masking effect on the

salticid clock was found under bright light, in which condition salticids were no longer able to anticipate lights-on and lights-off. Similar effects have been found in pigeons, *Columba livia*, at intensities of 2000 lux (Yamada et al. 1988).

Where observed, the free-running period of spiders changed direction depending on light conditions: under constant normal light, the period reduced to < 24 h, while under moonlight intensities, the period increased to > 24 h. Evidently, much is yet to be understood regarding the effects of light intensity on salticids, which should be addressed in the future in more natural conditions.

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Chapter 6

Effect of light intensity on the free-running period and entrainment of salticid spiders

Abstract

Experiments using abrupt changes in light levels are able to differentiate circadian-based anticipatory behaviour from direct responses. The organism to be tested must be transferred to what is called "constant conditions" to determine whether locomotor activity is only a response to the environmental change. With an increase in light intensity, the free-running period in many diurnal animals shortens, but lengthens in nocturnal species. In this study, I investigated Aschoff's rule using *Marpissa marina* (Araneae: Salticidae; Goyen, 1892) in conditions of constant darkness and constant light at different intensities. I found in *M. marina*, intense constant light induces hyperactivity, which disrupts rhythmicity, with only some exhibiting a weak rhythm during the first four days. Salticid oscillation system 'tries' to keep its rhythm in first days of exposure to bright light and in this situation, the free-running period tends to be greater than 24 h, while they show strong rhythmicity in constant dim light with a trend to lengthen the activity more than 24 h. This trend was the opposite in constant darkness (after day 10). The spiders lost their rhythm for more than a week under total darkness (masking effect), but returned to a shortened period of rhythmic activity after 10 days. Irrespective of light intensity, under constant light conditions, the free-running period was lengthened, as suggested by Aschoff's rule (1960) and now found in many taxa.

Keywords: lengthening, shortening, circadian rhythm, arrhythmic

Introduction

Circadian activity rhythms are part of the adaptations of a species to its environment, reflecting the adjustments in animals' physiology and behaviour to the diel changes in environmental conditions (e.g. Beltrán & Delibes 1994; Erkert & Kappeler 2004; Brown et al. 2011). These adjustments probably result from interactions between a number of exogenous abiotic and biotic factors (zeitgebers) and an endogenous biological clock or circadian pacemaker (Goldman 1999; Mistlberger & Rusak 2005). Circadian rhythms typically are entrained by environmental cycles, most

notably the daily alternation of light and dark, which is a virtually universal zeitgeber for the circadian rhythms displayed by most species (Daan & Aschoff 1975; Bertolucci et al. 1999), implying that animals that do not live on or near the equator may experience seasonal changes in day length that require flexible behavioural adjustments to their seasonal circadian activity rhythms (Erkert & Kappeler 2004). Entrainment ensures that the rhythm maintains a fixed phase relationship with the environmental cycle and activates rhythmic outputs at the appropriate time of day (Hardin 2005). Often, the entrained rhythm makes it possible for the organism to anticipate environmental changes, such as sunrise or sunset. The entrained circadian rhythm thus may allow the organism to be proactive rather than reactive with respect to daily periodic environmental stimuli. For example, in the field, honeybees, using their circadian clock-driven time-memory, anticipate the time of day at which they encounter profitable food sources on previous days by scheduling reconnaissance flights to that particular location in the environment, with the amount of anticipation depending on the amount of previous experience with that source (Moore & Doherty 2009).

Experiments using abrupt changes in light levels are able to differentiate circadian-based anticipatory behaviour from behaviours that respond directly to (gradually) changing light levels. For example, the entrained circadian rhythm of activity in bats (DeCoursey 1964) and squirrels (Hut 1999) allows these nocturnal animals to anticipate sunset, thereby enabling them to begin their night-time activities at the appropriate light intensity level. However, to establish a periodicity as an endogenous one, it is necessary to exclude all possible zeitgebers. Therefore, the organism to be tested must be transferred to what is called "constant conditions" to determine whether locomotor activity is only a response to the environmental change. In this manner, animals have been studied in constant darkness (DD) and in constant illumination (LL) such that behavioural cycles are displayed spontaneously if reliant on internal sources. Such cycles are by their regularity often attractive as models for the analysis of temporal organization (Daan et al. 2001).

In keeping constant all variables of the environment most commonly controlled, such as light, and temperature, three results of such an experiment are possible. Firstly, the periodicity may suddenly stop or damp out within a few periods. This result is not convincing proof for or against an endogenous period. It could be the case that a periodicity, although endogenous, becomes unobservable by the tested function

because the environmental factors chosen for constancy are too unfavourable (environment too hot, too cool, too bright, too dark, etc.). Secondly, the periodicity might continue with a period of 24 hours. In this case, one can not exclude that an overlooked or unknown periodic factor of the environment was effective as a zeitgeber. Experiments in constant conditions, the results of which show an unaltered frequency and phase of the organism, do not prove an endogenous periodicity, and still less that this periodicity was inherited. Finally, the periodicity might continue, but with a frequency deviating by a certain, more or less constant, amount from that of the Earth's rotation. If there is no other periodicity in the environment (perhaps a tidal one), with which the organism is in synchrony, then the periodicity is really endogenous (Bordyugov et al. 2015; Aschoff 1960; Bruce1960). The free running period is the only convincing evidence of an endogenous (circadian) periodicity. Organisms in constant conditions may retain unaltered phase relationships with the external physical cycles for periods of up to a month (Aschoff 1963) and its why the scientists say; free-running period is history dependent.

Aschoff (1960) showed a relationship between the behavioural rhythmicity of the animals under DD or LL and their activity patterns, whether they be predominantly diurnal or nocturnal. According to his 'rule', nocturnal animals continue their rhythmic activity under DD, while in LL they become arrhythmic. Aschoff (1981) believed that in most species, the free-running period (circadian period) depends on the intensity of light, showing that, with an increase in light intensity, the free-running period in many diurnal animals shortens, but lengthens in nocturnal species (Aschoff 1981). Spiders appear to rely on circadian clocks to regulate a variety of behaviours and physiological processes (Seyfarth 1980; Schmitt et al. 1990; Suter 1993; Yamashita & Nakamura 1999; Ortega-Escobar 2002; Jones et al. 2011). Circadian clocks control locomotor activity (Hardin 2005) and have been studied in spiders both in the field (diel rhythms) and in the laboratory (diel and circadian rhythms) (Ortega-Escobar 2002). In this study, I investigated Aschoff's rule using spiders from the family salticidae in conditions of DD and LL at different intensities. Specifically, the questions addressed here were:

- Is the free-running period in *M. marina* affected by light intensities?
- Are *M. marina* spiders able to keep a diel (circadian) rhythm under total darkness and illumination?

- How does light intensity influence the onset of activity in *M. marina*?

Being diurnal, I expected *M. marina* to exhibit a shortening of the free-running period under LL. I also expected that in very bright constant light they would not show significant rhythm of circadian activity due to a masking effect. Also, as these spiders are so visual, I expected to find that constant darkness would mask their circadian rhythm similarly. The most important side of this study is to interpret and analyse the rhythm and activity at the weak point that the other studies just have referred to that as 'non-significant'.

Methods

Twenty field-collected (as juveniles) virgin adult female *Marpissa marina* (Araneae: Salticidae; Goyen, 1892) were used for all experiments. Daylight intensity in the microhabitat (around rocks and coastal algal debris) of *M. marina* was measured as 100 lux by means of a light-meter (LI.COR, model LI-250), which was used as the 'normal' intensity in my experiments (L_n). High brightness on a sunny day in their habitat was measured at between 300 to 400 lux, and the minimum brightness in their habitat (under stones) was 7-9 lux. Here, I considered 300 lux as intense (bright) illumination (L_i) and 9 lux as dim light (L_d).

For maintenance, spiders were individually-housed in plastic containers (40 mm diameter × 50 mm high) with a ventilation hole covered with mesh and a hole at the bottom containing a cotton wick which hung into a glass of water to provide humidity and drinking water (Jackson & Hallas 1986). An additional hole, plugged with a cork, was used for feeding the spiders with house flies, *Musca domestica* L., once per week. The animals were released following experiments. All spiders were fed to satiation on house flies 24 h prior to testing (i.e., there were always live prey remaining in the cage after 24 h). During the 20 days of total darkness, the spiders were fed under red light every 5 days. Data from feeding days were removed from the results. For measuring locomotor activity all methods were as described in Chapter 2. Here, I ran four sets of experiments three of which began with a short LD (three days) followed by different

light conditions; the fourth began with five days of DD. I have previously determined that three days is sufficient to observe changes in activity patterns so I considered three days for light:dark cycles for entrainment and up to 20 days for constant conditions to be ample to determine the effects of light intensity on the free-running period. To maintain spider health, prior to the constant light conditions, spiders were removed during 'light' and placed in their cages for 4 h and were fed a housefly before being returned, still during 'light', to clean glass tubes and replaced in the locomotor activity monitor (LAM). No further feeding of the spiders was done in constant conditions in order to minimise the impact of this on their behaviour. All spiders were returned to the channels within the LAM in which they had been previously placed.

The first experiment started with a normal, entraining, L_nD 12:12 condition for three days followed by 20 days under constant intense light (L_iL_i) (Fig. 1a), with 17:30 being considered as ZT0 for measurements (start time). The second experiment was identical except that I constant dim light, L_dL_d, instead of intense LL (Fig. 1b). The third experiment changed only that after the entraining period, spiders were exposed to (DD) for 20 days (Fig. 1c). For the last experiment, spiders were fed after a five day DD condition and were introduced to an intense light/ dark cycle (L_iD) for four days followed by another five days of DD and then by a dim light/ dark cycle(L_dD) for a final four days (Fig. 1d). Although, these LD cycles were initially 6 days, the first 2 days after both of them were considered as transient days (the cycles that spiders needed to adjust their body clock) and were eliminated from the results.

For analysis, 30 min bins were used for activity data. Passing the spiders through the test tube and from the central infrared beams counted as one unit of activity which reported every minute by the LAM. I used the ActogramJ plugin in ImageJ to determine the slope of onset of activity across 24 h periods using Chi-square and Lomb-Scargle analyses (and Fourier for not significant ones). The results of the periodogram were then submitted to a statistical test suggested by Siegel (1980). In this method, the significance of contemporary frequencies recognised by the periodogram is tested against the null hypothesis that the signal is indiscoverable from a fortuitous association of oscillations (white noise). This method has the advantage of testing the significance of both the overcoming and other oscillations, displaying an amendment of Fisher's test of significance in rhythmic analysis. If a period was significant, I used Lomb-Scargle to analyse the peak and find the precise tau factor (free-running period). The orientation

of rhythm is a characteristic which is measurable by means of last or next LD and is more reliable when measured at an individual level (because of large inter-individual differences in responses). In the cases of weak rhythmicity, the lower limitation of activity adjusted to 7 in ActogramJ to get rid of the noises and tiding the rhythm up. Also, when the level of activity was not assignable, the upper limitation increased to accentuate the actogram. As there was large individual variation, I depict periodograms belonging to 'typical spiders', defined as those that characterise 60-70% of the tested population.

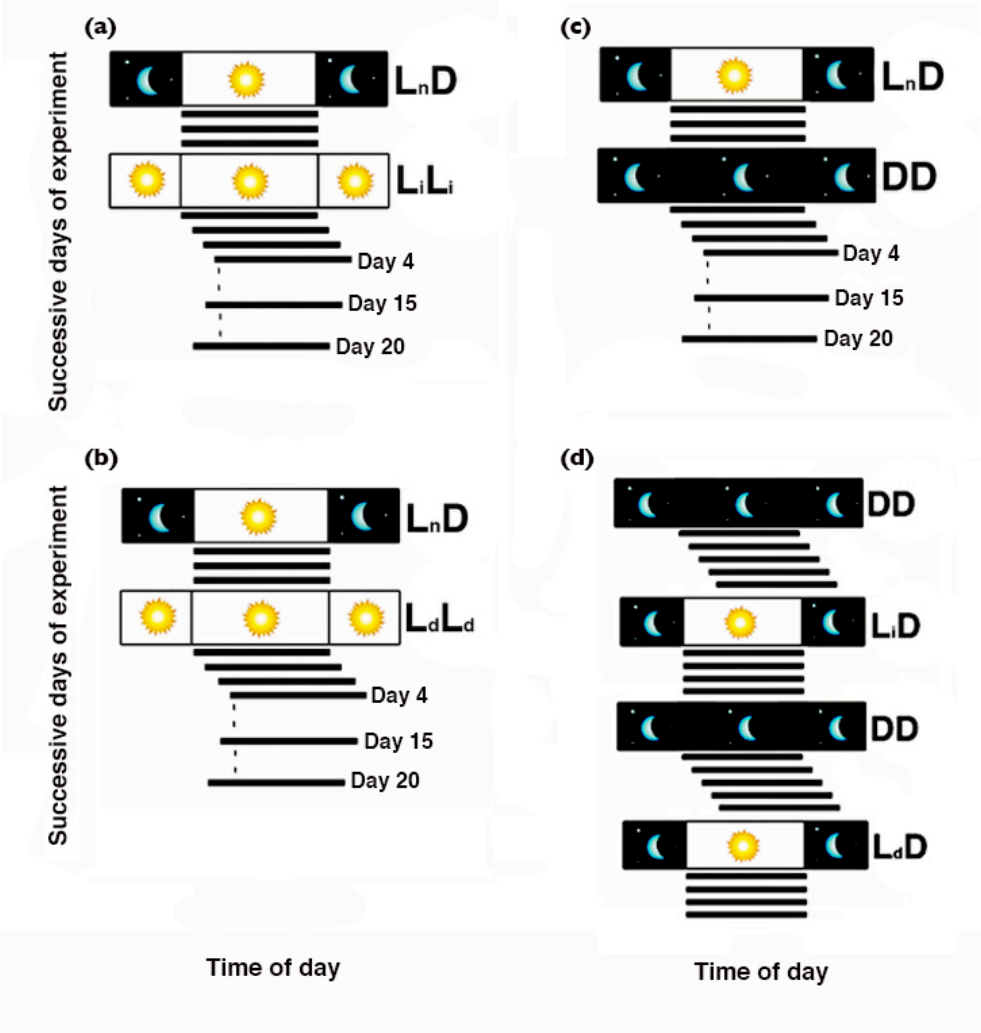

Fig. 1. Experimental protocol for determining the effect of light intensity on *M. marina* (Salticidae) free-running periods. For three of the four experiments a 3-day LD (light/dark) (12:12) cycle (with normal light intensity) was followed by constant conditions. **a.** Normal light/dark condition (L_nD) followed by 20 days under constant intense light (L_iL_i). **b.** Normal light/dark condition (L_nD) followed by 20 days under constant dim light (L_dL_d). **c.** Normal light/dark condition (L_nD) followed by 20 days under constant darkness (DD). **d.** 5-days constant darkness, followed by four days under L_iD with intense light/dark cycles and another 5-days under constant darkness, followed by another light/total dark, but with dim light (L_dD). Horizontal black bars depict activity over successive days.

Results

Under constant intense light (L_iL_i), about 75% of spiders showed an arrhythmic locomotor activity from the first day to day twenty, and the rest (25%) displayed either right to left (<24h) (34%), or left to right (>24 h) (66%) free-running patterns, but only in first four days (Fig. 2a, b), after which there was no rhythm and the spiders displayed hyperactivity (high activity not seen in spiders in normal light conditions) (Fig 2c). Of the 25% of spiders that displayed weak rhythmicity in the first four days, none showed significant periodicity (Fig. 3a, b) although the rhythm was stronger for spiders exhibiting >24 h patterns (Fig 3c, d), with peaks of activity typically between 22 h to 30 h according to subjective day. Irrespective of the orientation of the activity pattern, spiders during the first four days started their activity with a delay (before ZT6) (Fig. 4). Of the 15% of spiders that showed any rhythmicity from day 5 to 20, none began their activity before ZT0, instead starting their activity somewhere around ZT6, but none of them showed an advance of activity before or equal to ZT0 during 20 days of experiment (tau factor tended to be greater than 24 h) (Fig. 4b). The remaining spiders showed arrhythmicity.

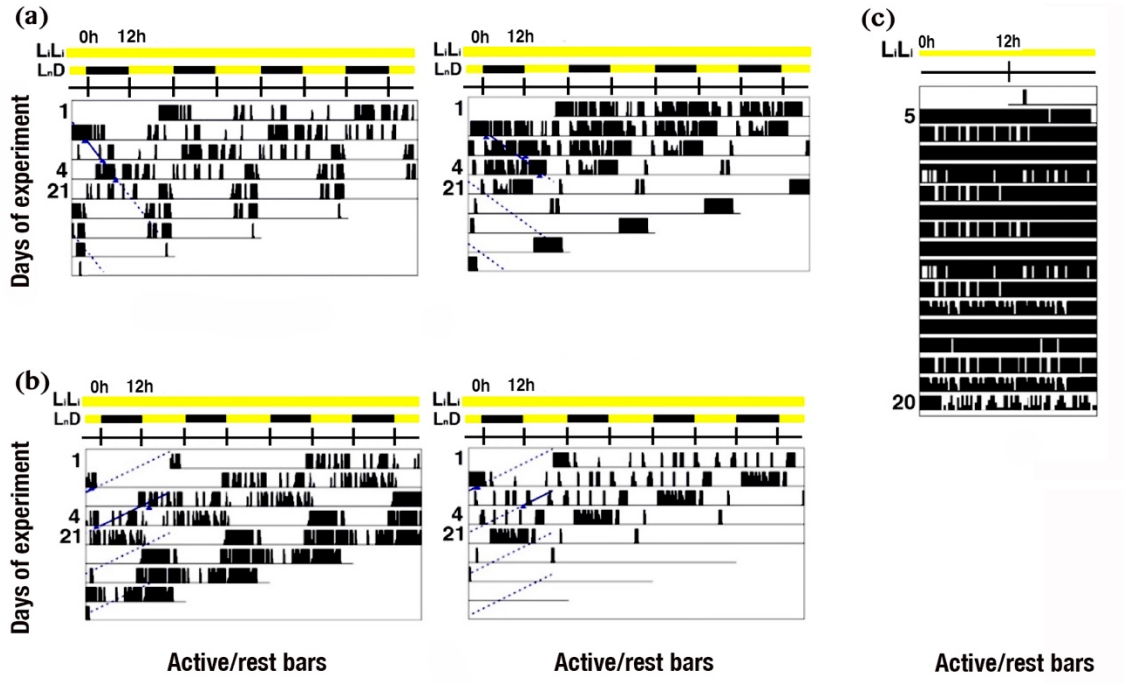

Fig. 2. Activity (black bars) under constant intense light (LiLi). The lower limit of ActogramJ was adjusted to 7 for presentation. **a.** Weak left to right free-running rhythmicity (66% of spiders displayed this) in 2 typical spiders (spiders which show the characteristics of most of the population; not too slow and not hyperactive) and **b.** Weak right to left free-running rhythmicity (34% of spiders showed this rhythm) in two typical spiders (four-plated actogram). For graphical representation, the following L_nD cycle is depicted right after day four, with the intervening period shown separately in panel c. **c**. Double-plated actogram of typical response of spiders from day 5 to 20. All the spiders showed arhythmicity and hyperactivity in this period.

For comparative purposes, the first four days under dim L_dL_d are initially described independently of the remaining days. In contrast to intense light condition, the actogram of spiders for these days showed a strong rhythm of left to right orientation (tending to be > 24 h) (Fig. 5a) in about 80% of spiders (the orientation was measured based on last LD) and the rest didn't show clear-cut rhythmicity. The mean (± SD) onset of activity after four days was seen after 14 h ± 1.3 (N = 20, n = 16), between ZT12 and ZT18. The orientation of activity from day four onwards remained on a left to right orientation and with onset of activity gradually increasing to 17 h ± 2.4 (N = 20, n = 16) latency (delay in onset towards light-on) by day 20 (Fig. 5b). The Chi-square periodogram of activity under L_dL_d showed a single peak of significant periodicity between 13.5 h and 16 h in 80% of spiders (first hours of their subjective day) (Fig. 6).

As the periodicity was significant under dim constant light, it was possible to measure the free-running period of these spiders by means of the Lomb-Scargle test. This analysis showed a free-running period of 42.30 h ± 3.8 (N = 20, n = 16) with a recognised dominant peak of activity.

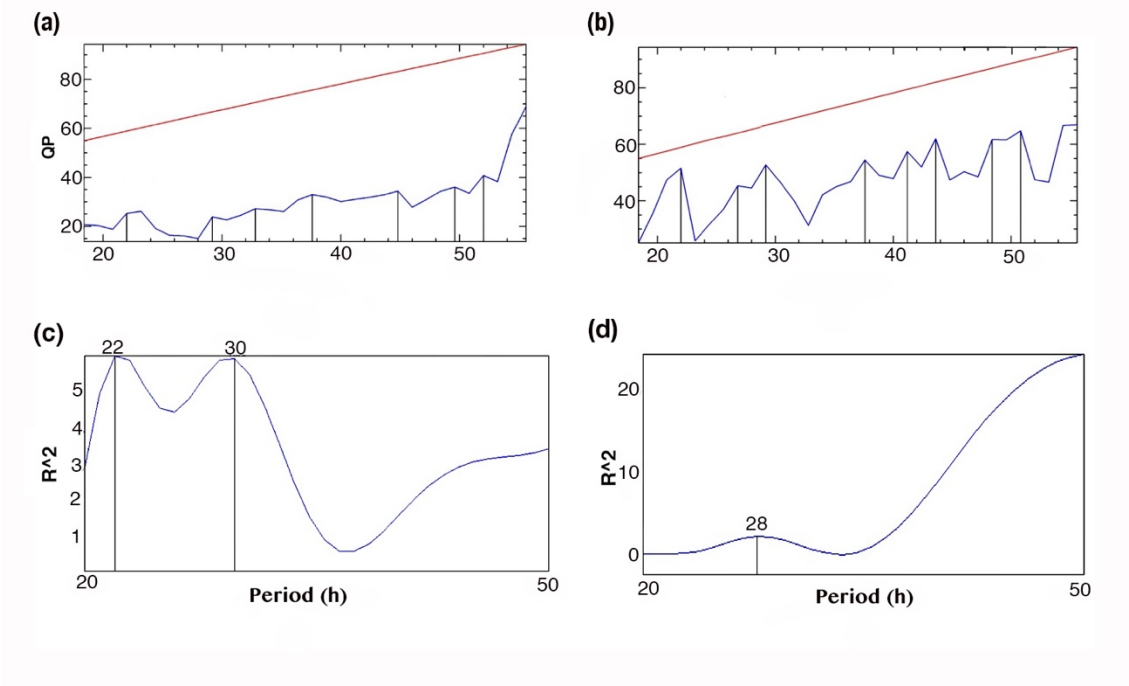

Fig. 3. The rhythm analysis for 4 days under L_iL_i for 2 typical spiders of *M. marina* (Salticidae). Chi-square periodogram under intense L_iL_i showed free running periods were not significantly rhythmic in right to left orientation (**a**) and left to right orientation (**b**). The red QP (value of Chi- Square periodogram for data set) line indicates the line of significance. **(c)** Fourier periodogram for a spider with right to left orientation. Activity started at R^2 (amplitude of period in Furrier periodogram) >3. The dominant peaks of activity happened at 22 h and 30 h (but not significantly). **d.** Fourier periodogram for a spider with left to right orientation. Activity started with R^2 > 0. The single dominant peak of activity happened at 28 h (but not significantly).

In constant darkness, during the first four days after entrainment all the spiders showed arhythmicity with low activity. From day 10 to 20 low levels of activity continued, but with a significant rhythm with a right to left orientation (i.e., tau < 24 h) for 85% of spiders (Fig. 6). The tau angle of difference was -2.45 h ± 1 (N = 20, n = 17) by day 20; thus, on day 20 the mean period length of free-running period was 21 h ± 1

($N = 20$, $n = 16$) (below ZT0). The Lomb-Scargle analyses showed a 21.46 h ± 1h ($N = 20$, $n = 16$) mean length of free-running period for the last 10 days under DD and the Chi-square periodogram of activity under DD showed a single peak of significant periodicity between 21.5 h to 23 h among individuals.

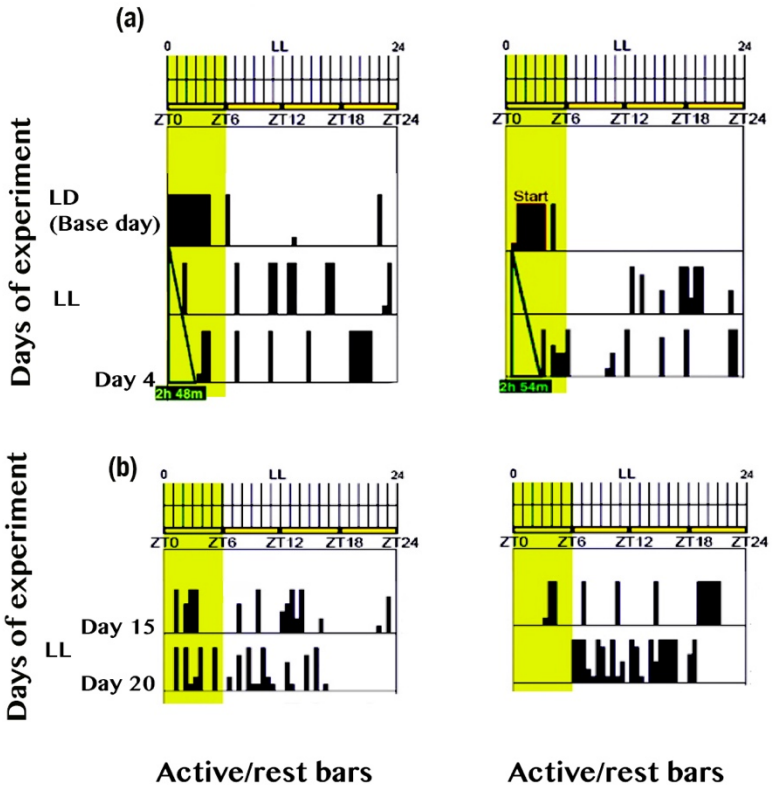

Fig. 4. The onset of activity in *M. marina* (Salticidae) under intense constant light condition. The green triangle is 'eye-fitted line tool' to measure the difference angle. **a.** All spiders, irrespective of rhythm orientation showed a delay in onset of activity between ZT0 and ZT6 during the four first days of the experiment. Here is the representative of two typical spiders with the delay around 3 h in day four. The last day of last LD considered as base for measurements. **b.** Between the day 15 and 20 of experiment, spiders started their activity between ZT3 to ZT8, but never started at ZT0 or before that. Two representative graphs of typical spiders in this category presented.

The rhythmicity under intense and dim light / dark condition showed that under L_iD more than 70% of spiders entrained to the new L_iD cycle after a five-day free-running under DD within an hour from the first cycle (as the free-running period is history dependent, I designed a DD before each entrainment to make sure that the LD

cycle was not influenced by any other stimulus before starting) and confined their activity to the light hours.

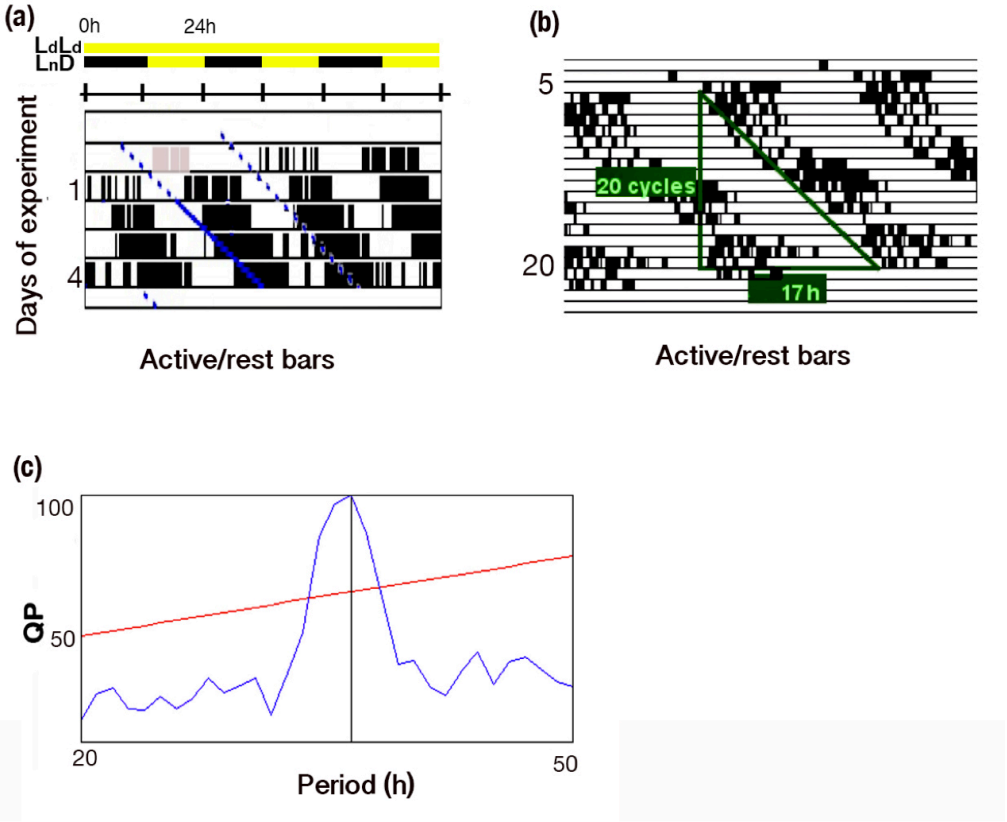

Fig. 5. Foursome plated actograms of activity depicted under constant dim light condition in *M. marina* (Salticidae). **a.** Orientation of rhythm in first four days in a typical spider. The blue lines show the orientation of free-running. About the 80% of spiders showed left to right activity patterns (>24 h). **b.** Mean tau at day 20 was of 17 h ± 2.4 for the same typical spider. **c.** Representative of average Chi-square periodogram for all the 20 days period in a typical spider. QP is the value of Chi-square periodogram for data set and the red line shows the significance of this period. 85% of spiders with rhythmic free-running period under constant dim light condition showed this periodogram.

However, the rest of spiders started their activity on the first day, with a mean delay of 2 h $\pm$ 0.20 ($N = 20$, $n = 6$), and re-entrained from the second day of experiment with zero difference angle toward the light- on time. After entering a new dim light / dark cycle (L_dD) from a five-day DD, no spiders entrained to the new cycle immediately from the first day of entrainment and they started their activity with a mean delay of 7 h $\pm$ 2.5 ($N = 20$).

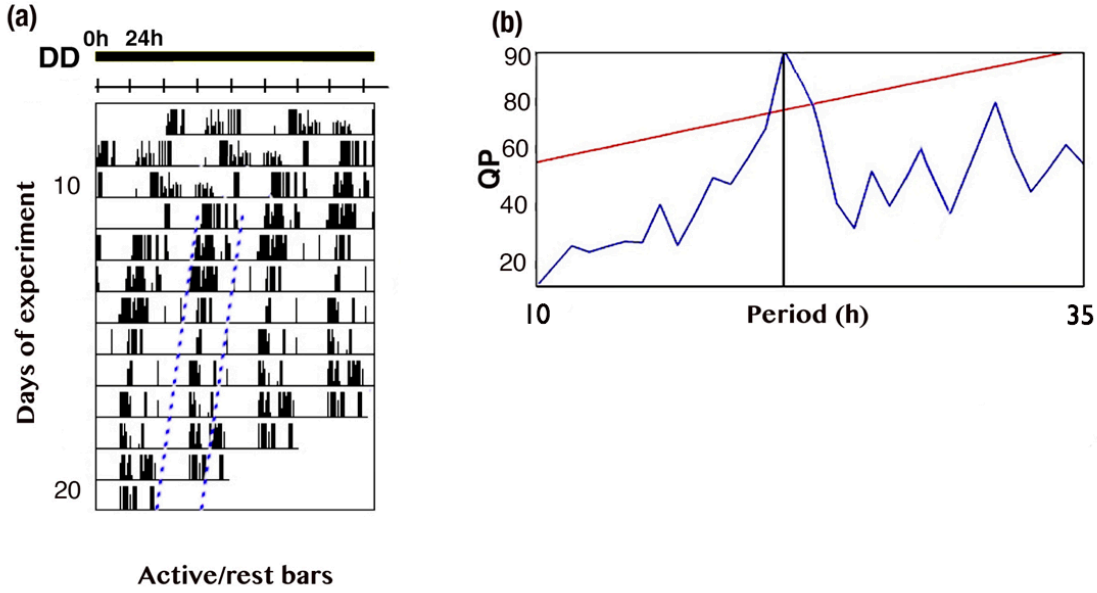

Fig. 6. Rhythm during the last 10 days of experiment under DD in *M. marina* (Salticidae). **a.** Actogram of free-running period under constant darkness for a typical spider. The orientation of rhythm from day 10 to 20 was right to left **b.** The average Chi-square periodogram under DD for the period of experiment for a typical spider. QP is the value of Chi-square periodogram for data set and the red line shows the significance of this period.

Chi-square periodograms for the spiders under L_iD, showed the significant peaks (one strong and one weak) during 34 and 36 h (Fig. 7a). A chi-square periodogram under dim L_dD condition showed the single peak between 45- 49 h and higher activity levels of activity (Fig. 7b). When activity was plotted into five time bins (2 h each), results were comparable in terms of the difference of activity during the day (Fig. 7c, d).

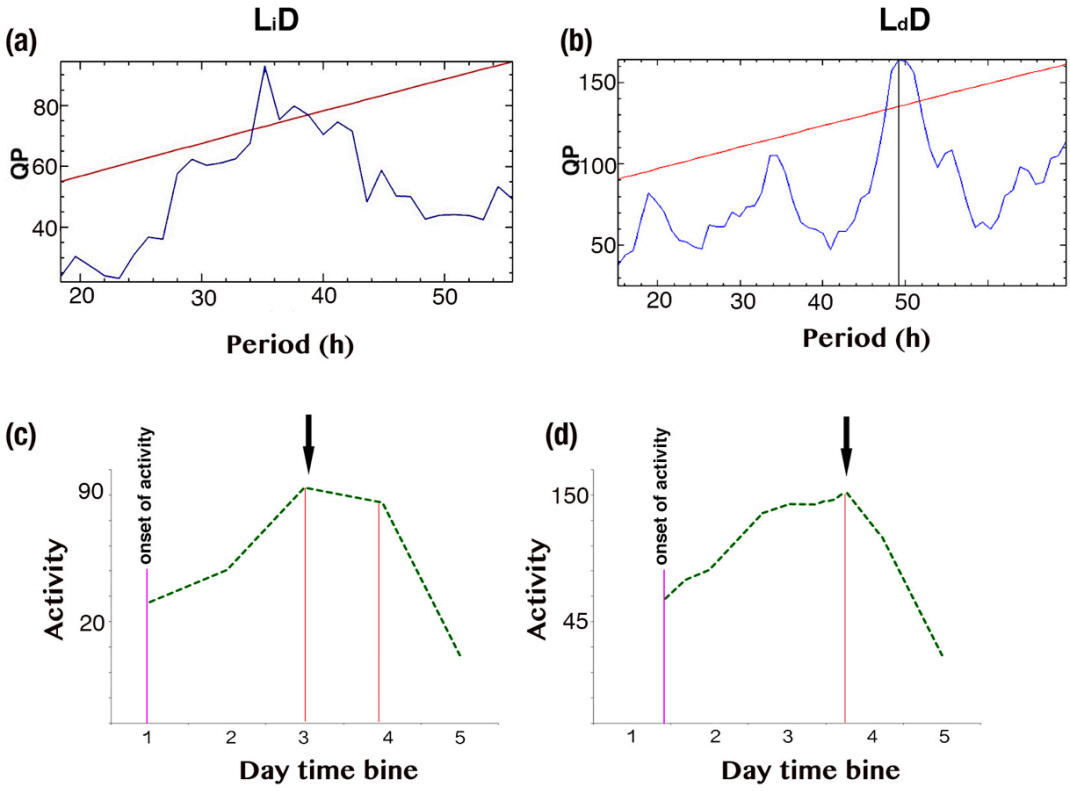

Fig. 7. Analysis of rhythm in second day of entrainment under intense (L_iD) and dim (L_dD) cycle in *M. marina* (Salticidae). **a.** Chi-square periodogram for a typical spider showed significant periodicity in 34 h and 36 h under intense LD. **b.** Chi-square periodogram for the same spider showed significant mono-peak periodicity at 49 h under dim LD. QP is the value of Chi-square periodogram for data set and the red line shows the significance of this period**. c.** Individual representative of activity in five time bins of the day for the same spider under intense LD and (**d**) under dim LD. The onset of activity in both the five-time bin graphs is comparable.

Discussion

Depending on the intensity of light, constant light is able to disrupt circadian rhythmicity in salticids, with a variety of responses exhibited, ranging from reduced locomotion and an increase in tau to complete arrhythmicity (Pittendrigh 1976, Aschoff 1981), as seen in the beetle *Carabus violaceus* showing arrhythmicity during the Arctic summer (LL) (Hempel & Hempel 1959). I found in *M. marina*, intense constant light appears to induce hyperactivity, which disrupts rhythmicity, with only some exhibiting

a weak rhythm during the first four days. Similarly, Ortega (2010) showed that, under constant light, nocturnal spiders of family Lycosidae showed arrhythmicity. Chen et al. (2008) examined what happens in mammals when, after chronic LL treatment, animals are put back into constant darkness, finding that upon transfer to DD, circadian rhythms in both behaviour and gene expression were very quickly regained and started from a specific phase, suggesting that the clock output was masked by the constant intense light treatment. However, what constitutes intense light may differ depending on an animal's life history, with light intensity causing arrhythmicity being higher in diurnal animals than in nocturnal animals (Pittendrigh 1958). Here, I also investigated the trends of non-significant rhythms, finding that the salticid oscillation system 'tries' to keep its rhythm in first days of exposure to bright light and in this situation, the free-running period tends to be greater than 24 h. While spiders became arrhythmic under constant intense light, they showed strong rhythmicity in constant dim light, which is reminiscent of results in *Drosophila pseudoobscure* under constant intense light (Chandrashekaran 1998), with differences in that dim light in salticids is considered intense for *Drosophila*.

The trend of activity under constant dim light in salticids was left to right (> 24 h), but was the opposite in constant darkness (after day 10). Aschoff's 'rule' (1960) posits that nocturnal animals should show a free-running rhythm under DD and be arrhythmic in LL. As salticids are diurnal, I expected them to show arrhythmicity under DD. However, my results suggest total darkness may act as a masking factor on the spider's clock, such that they their lost their rhythm for more than a week, but returned to a shortened period of rhythmic activity in a rhythm after 10 days. In the nocturnal Lycosidae, spiders displayed a free-running rhythm under DD with a single peak (Ortega-Escobar 2002). In the diurnal *M. marina*, I found arrhythmicity (i.e., being active virtually through the entire 24 h) under intense LL with no significant rest phase. In contrast, nocturnal lycosids under LL show a very marked inhibition of locomotor activity (Ortega 2010).

Under constant dim light, there was a delay in the onset of activity compared with constant intense light, further supporting the notion that under dim light, the length of the free-running period in *M. marina* tended to be greater than 24 h. In addition, a lengthening of the free-running period was seen under the very first days of intense constant light such that, in contrast to intense darkness, irrespective of light intensity,

under constant light conditions, the free-running period was lengthened, as suggested by Aschoff's rule (1960) and now found in many taxa, including fish (Tabata 1992), reptiles (Tosini 2001), birds (Aschoff 1967), and mammals (Carpenter & Grossberg 1984), with diurnal species tending to extend their period with increasing light intensity (Aschoff 1981).

In contrast with my results, Aschoff (1981) showed circadian periods under constant darkness for 11 species of diurnal animals were above 24 h (between ZT0 and ZT6). However, there is considerable variation among animals. For instance, in *Coccinella septempunctata bruckii* Mulsant (Coleoptera: Coccinellidae) rhythmic patterns with a free-running period of 22 h persisted under LL (Nakamuta 1987). Similar to my results, the millipede *Blaniulus lichtensteini*, under DD conditions showed brief rhythmic sequences, with a period of less than 24 h (Mead and Gilhodes 1974).

The free-running period (i.e., the period of the circadian rhythm), is specific to each species and is set to environmental phenomenon such that any disruption in this may lead to death of animal. For instance, Spoelstra et al. (2016) showed that mice with a tau mutation leading to shorter circadian periods showed reduced survivorship and reproduction relative to wild-type mice when released. I found in intense light (but not dim light) after a period DD *M. marina* illustrated two peaks in their active phase, but was unable to find this under constant intense light conditions over a longer period because spiders became hyperactive, which would like lead to death over extended periods of time. Aschoff (1954, 1957) noted many decades ago that the daily activity pattern of animals commonly includes two distinct components. These are often clear, but in some cases, the two peaks are not clearly distinct when the animal is very active throughout its activity time (α), as I found with *M. marina*. Altogether, my results suggest that the circadian oscillator(s) of *Marpissa* has (have) a robust organization which is strongly affected by light intensity.

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Chapter 7

Pathways of ocular entrainment in *Marpissa marina* (Araneae, Salticidae)

Abstract

Depending on animal species, photoreceptors are locate in the visual organs, in non-visual organs or in both. Because of unique characteristics of vision containing several different pairs of eyes, I studied the jumping spider (salticid) *Marpissa marina* (Araneae: Salticidae; Goyen, 1892). Eyes in spiders are categorized in two groups of principal and secondary. Specifically, my aim was to determine which eyes dedicated to regulation of the central circadian rhythm and to illuminate the pathway (s) of ocular entrainment in jumping spiders. To prevent entry of light to the photoreceptors, I used an opaque elastic paste. My procedure was to measure spider activity level over eight days, and their responses to a 6 hour delay in light/dark cycle, using spiders with uncovered eyes and then with distinct pairs of eyes covered. The results revealed that, unlike the secondary eyes, light information gathered through AM eyes did not lead directly or indirectly to the parts of the circadian system that contain circadian pacemakers.

Keywords: Circadian rhythm, entrainment, jumping spiders, eye function, photoreceptors

Introduction

A fundamental rhythm in animal behavior is the circadian rhythm of daily activity (Bradshaw & Holzapfel 2010), whereby an external signal, such as day and night, acts as an entraining 'zeitgeber' (translated as 'time giver') for the internal oscillator (Rensing & Ruoff 2002). This circadian system should include a photoreceptor(s), in order to synchronize with environment, which depending on animal species, is located in the visual organs, in non-visual organs or in both. The pathways of entrainment have been investigated in mammals (Moore 1995; Dibner et al. 2010; Gooley & Saper 2017), insects (crickets, Tomioka & Yukizane 1997; Tomioka

2014, *Drosophila,* Helfrich-Förster et al. 2001; Yoshii et al. 2015, cockroaches, Nishiitsutsuji-Uwo & Pittendrigh 1968; Homberg at al. 2003, scorpions Fleissner & Fleissner 1993) as well as in the family of lycosid (Ortega-Escobar 2002) spiders.

Animals have either single-chambered, camera-type, eyes or compound eyes. Spiders have camera-type eyes (Land 2005). Most spiders are nocturnal, but the family Salticidae (jumping spiders) are diurnal hunters. In terms of functional and anatomical grounds, eyes in salticids are divided in two groups: the principal and the secondary eyes (Land 1969, 1985). The principal eyes or antero-median eyes (AM eyes) consist of a single pair of forward-facing eyes with movable retinae at the end of a long cone. The anterior lateral eyes (AL eyes), posterior median eyes (PM eyes) and posterior lateral eyes (PL eyes) are the secondary eyes. These have a simple, single-layered retina which does not move. All eyes, except AM eyes have a tapetum that reflects light after it has passed through the receptors. The rhabdoms of the AM eyes are placed between the cell body and the lens; while in the secondary eyes the cell body is placed between the lens and the rhabdomere (Kovoor et al. 1993; Homann 1971).

Spider eye arrangement may be altered in different families of spiders. In salticids (Fig. 1), the AL eyes sit just beside and behind the AM eyes. There is a huge reduction in size in PM eyes which are above and a bit behind the AL eyes. The PM eyes are vestigial in many species. By blinding the secondary eyes, Homann (1928),Crane (1949) and Forster (1979) showed that these prevent orienting towards stimuli which are out of the field of view of principal eyes, meaning that these are principally motion-detectors (Land 1985, Zurek et al. 2010), but blinding the principal eyes prevents visual accuracy. In the family Lycosidae, the eight eyes are arranged in three rows. The front row is composed of the AL eyes and AM eyes. The middle row comprises the PM eyes; and the posterior row, the PL eyes. In comparison, in the family Lycosidae (*Lycosa tarantula*), AM eyes function for polarized-light orientation (Ortega-Escobar 2017; Ortega-Escobar & Muñoz-Cuevas 1999) while the AL eyes, PM eyes, and PL eyes function for LD cycle entrainment (Ortega-Escobar 2002).

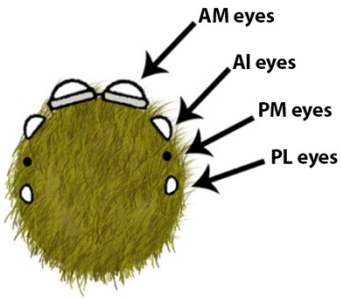

Fig. 1. Representation of principal and secondary eyes in jumping spiders. The large AM eyes in front of the prosoma are considered the primary eyes. The AL eyes, PM eyes and PL eyes are called secondary eyes.

The daily rhythms of most organisms are synchronised to a period of 24 hours, a process called entrainment. The clock entrains using environmental cues called zeitgebers which can include light, temperature and social activity (Turek & Losee-Olson 1987). If the organism has photoreceptors with entrainment tasks, while exposing photoreceptors to a change in light regime (a shift in light hours), it is expected that the organism will re-entrain to the new LD cycle. I used this to simulate shift hours in an incubator. With separate experiments of eye covering, I examined the ability of spider`s eyes (pair by pair) to re-entrain to a new LD. If the onset of activity in spiders after the shift (apart from transient phase immediately after a light shift) was close enough to the light-on time (1 to 2 hours) in a few successive days, I considered that as entrainment. I translated the spider`s locomotion as index for their activity and counted their locomotor activity as the unit for spiders' response to light. I used the salticid *Marpissa marina* (Araneae: Salticidae; Goyen, 1892) as my test subjects. Specifically, my aim was to determine which pair of eyes (AM eyes, AL eyes, PM eyes, PL eyes) is responsible for entrainment if indeed the entrainment pathway is ocular.

One way of determining the response of circadian rhythm of an organism to environmental changes is to study the animal under constant conditions (Ortega-Escobar 2002), but to determine whether the entrainment pathway is ocular, one of the ways is covering the eyes to prevent entry of light to the photoreceptors which is the same condition as keeping the organism in constant light or dark (i.e., free-running

which is the situation that the rhythm persist even under constant darkness, but with period of > or < 24 h ; Vitaterna et al. 2001) if the eye in question was responsible for entrainment; for diurnal animals we would thus expect them to keep the rhythm in total darkness (DD) (Aschoff 1960) or when they eyes contain photoreceptors are covered. As the index to determine the existence of entrainment, the phase difference angle measurement was used. Pittendrigh & Daan (1976) define the phase angle of entrainment as the relationship between the timing of the biological clock and the timing of an external time cue (which is light-on in these tests). Smaller phase angles represent stronger entrainment if there is no masking effect or the activity is not under the control of hourglass clock (in both situations, the onset of activity is likely more immediate rather than entrainment; Mrosovsky 1999; Biebach & Krebs 1991).

Methods

Eleven field-collected (as juveniles) virgin, adult, female, *M. marina* were used for all experiments (four experiments were done for 4 pairs of eyes with the same spiders (9 treatment spiders as well as 2 control spiders). These spiders came from populations located in seaside areas of Christchurch, New Zealand (Birdling`s flat, Lat: -43° 49' 27.89" S, Long: 172° 42' 20.70" E) among the sands and stones, in summer. Immature specimens completed their development in the laboratory. For maintenance, spiders were individually-housed in plastic containers (40 mm diameter × 50 mm high) with a ventilation hole covered with mesh and a hole at the bottom containing a cotton wick which hung into a glass of water to provide humidity and drinking water (Jackson & Hallas 1986). An additional hole, plugged with a cork, was used for feeding the spiders with house flies, *Musca domestica*, once per week. The animals were released following experiments. All spiders were fed to satiation on house flies, 24 h prior to testing (i.e., there were always live prey remaining in the cage after 24 h).

To measure locomotor activity spiders were transferred individually into glass tubes (outer measurements, 16 mm diameter, 100 mm long; TriKinetics, Waltham, MA, USA) to measure locomotory activity. These were enclosed at one end by a snug-fitting small glass tube containing water plugged by a cotton wick that was inserted 10 mm into the locomotory activity tube in order to provide spiders with water throughout the

experiment. Locomotory activity tubes were enclosed at the other end by mesh held by a rubber band. Activity tubes were loaded into specially-designed locomotory activity monitors (LAM) (LAM25, TriKinetics, Waltham, MA, USA) enabling simultaneous recording for up to 32 channels (individual tubes). As each spider moved back and forth in its activity tube, it interrupted one of three infrared light beams that bisected the tube. Each crossing was counted by the system as one unit of activity and the activity counts per min for each tube were sent through an interface unit (PSIU9, TriKinetics, Waltham, MA, USA) via USB to a computer. Data were pooled into 30 min bins using the dedicated activity monitoring software (DAM File-Scan, TriKinetics, Waltham, MA, USA) for further analysis. Monitors were housed inside environmental chambers (Contherm-POLAR1000, 50 cm high X 50 cm wide X 35 cm deep; internal diameters) at a controlled temperature (22º C). Light was provided by a series of four strips of 12 wide-beam LED lights purpose-built for the chamber and placed on the inner ceiling to provide consistent lighting throughout the chamber. Nine spiders were used in eye covering treatments, with an additional two spiders being used as controls as one spider with no eyes covered and another with sham cover (with transparent substances were used to cover the eyes). Four treatments were done on the 11 spiders, testing each of the four pairs of eyes such that all eyes were covered except the pair in question. I used 9 lux as the light intensity based on my preliminary studies, which showed that they entrain very well to even low intensities of light. Low light intensity was applied to reduce the risk of passing the light through the eye-coats. The spiders were fed in their cages after each experiment and before the next experiment, which took place after one a day 'rest' period. After each experiment, I checked the eye-coatings; if any of them was taken off or had slightly peeled, the results of that spider were not considered. Covering the eyes was taking place in two days before clock shifts, under light hours and before starting the dark cycle. There were five days at the initial LD 12:12 (lights-on: 14:00 h and at 22º C) with no eye coverings (first two days were excluded as transition phase). Eye cover took place two days before clock shifts and after a one-day rest period in the same LD 12:12, the eyes covered spiders submitted to a new LD 12:12 cycle with light-on at 14:00 during five days. The first two days of this new LD were considered as transition phase and were excluded. Such that, just 3 days of each LD cycle was depicted and measured.

To cover the eyes, I used an opaque elastic paste which prevented sacrificing the spiders as this could be removed without harm after each treatment. Additionally, eye-covering has been proven as effective as interventionist testing, such as severing optic nerves (Ortega-Escobar 2002). The mixture that I used was based on Zurek et al. (2010). The base mixture contained 3D painting black paste (Sullivans), black ink (Parker) and a dental paste comprised of a base and catalyst (Coltene Whalendent®) which were mixed in a small funnel-shaped jar. After putting a tiny drop of the mixture on the target eye, I applied a small drop of white base paste (Coltene Whalendent®) on top and again added another drop of the dark mixture such that, the base paste acted as the cement and made the two layers of the mixture dried and firm (Fig. 2). To apply the drops on the eyes, the spiders were restrained in a 'gunkatron' which was made from two vials one of which fitted snugly within the other. The outer vial contained holes which were used to get access to the spider's cephalothorax. The inner vial had a sponge on the top to prevent the spider from being compressed against the outer vial. It took one minute for the mixture to dry, after which the spiders were transferred to the test tubes and the LAM monitor for activity measurement. At the end of each eye covering treatment, the spiders were again transferred to the 'gunkatron' to remove the eye covers with fine forceps. The placing of the covers was checked before removal; after removal they were also checked under a stereomicroscope (40x) to ensure that the entire surface of the eye had been covered (i.e., the elastic mixture was a full, dark, concave circle). In two experiments (covering the AL and PL eyes), the eye-coating of two spiders fell off, so I removed the results belonging to those spiders from entire data set, such that I analysed data for 9 spiders under all 4 eye treatments.

The period was analysed using Lomb-Scargle periodograms, (combined with a Chi-square test with 5% significance level) (Sokolove & Bushell 1978) in "ImageJ" software (Actogram J plugin) (Schmid et al. 2011). To determine the phase angle, eye-fitted line tools in ImageJ were used wherever needed which is the green triangle tool for measuring the difference phase angle between the onset of activity and any given light-on time in any cycle. In addition, to calculate the average phase difference of onset of activity toward light-on time in spiders, the average of difference time between the first reported activity in light cycle or subjective day toward its light-on time was measured. Paired two-tail t-tests were used to compare the mean activity level of treatments per day (for five days) with the control spiders and one-way repeated

measures ANOVA was used when comparing overall group treatments as well as Bonferroni post-hoc test whenever it was needed to test for significant differences between pairs of eyes. The figures were drawn in Prism software. All measurements are means (+/- Standard Deviation).

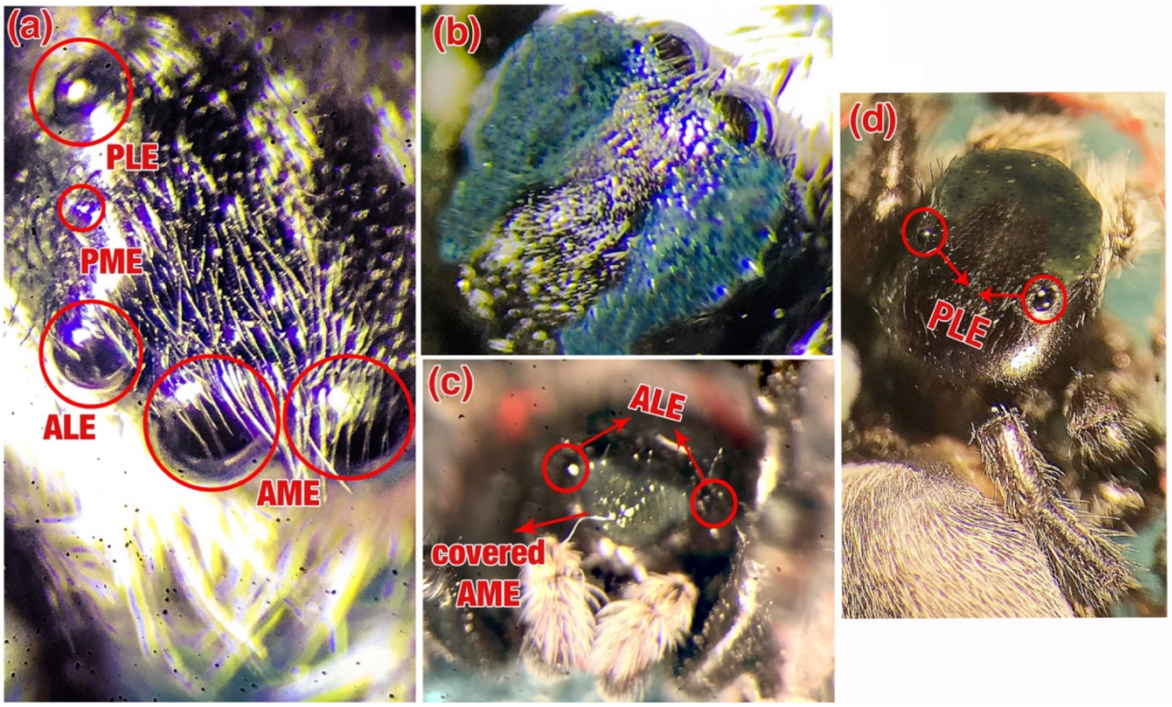

Fig. 2. Examples of covering eyes by means of the elastic mixture. **a.** View of the of eyes in *Marpissa marina*. **b.** All eyes covered except the anterior-median eyes (AM eyes). **c.** All eyes covered except the anterior-lateral eyes (AL eyes) **d.** All eyes covered except the posterior-lateral eyes (PL eyes).

Results

With the AM eyes uncovered (i.e., all secondary eyes were covered) the spiders continued their normal activity patterns on the fourth day. In day five, they were not entrained and showed a free-running with a trend of > 24 h. The phase angle of onset of activity in day five (the day after secondary eyes covering) towards the light on time (in the first LD) was of 1.07 h ± 0.19 (Fig. 3a). After a phase delay in day six, the test spiders did not entrain to the new LD regime and continued free-running in the opposite direction of the shift (lengthening the period; >24 h) such that, the period of free-

running on the last day of new LD was of 25.8 h ± 0.66 , $N = 11$, $n = 9$ (Fig. 3a, right) which was statistically significant at alpha = 0.05 (Fig. 3b). The control spiders with all the eyes uncovered and covered by sham cover showed a clear entrainment to the new LD after a 6 h phase delay-shift (Fig. 3c).

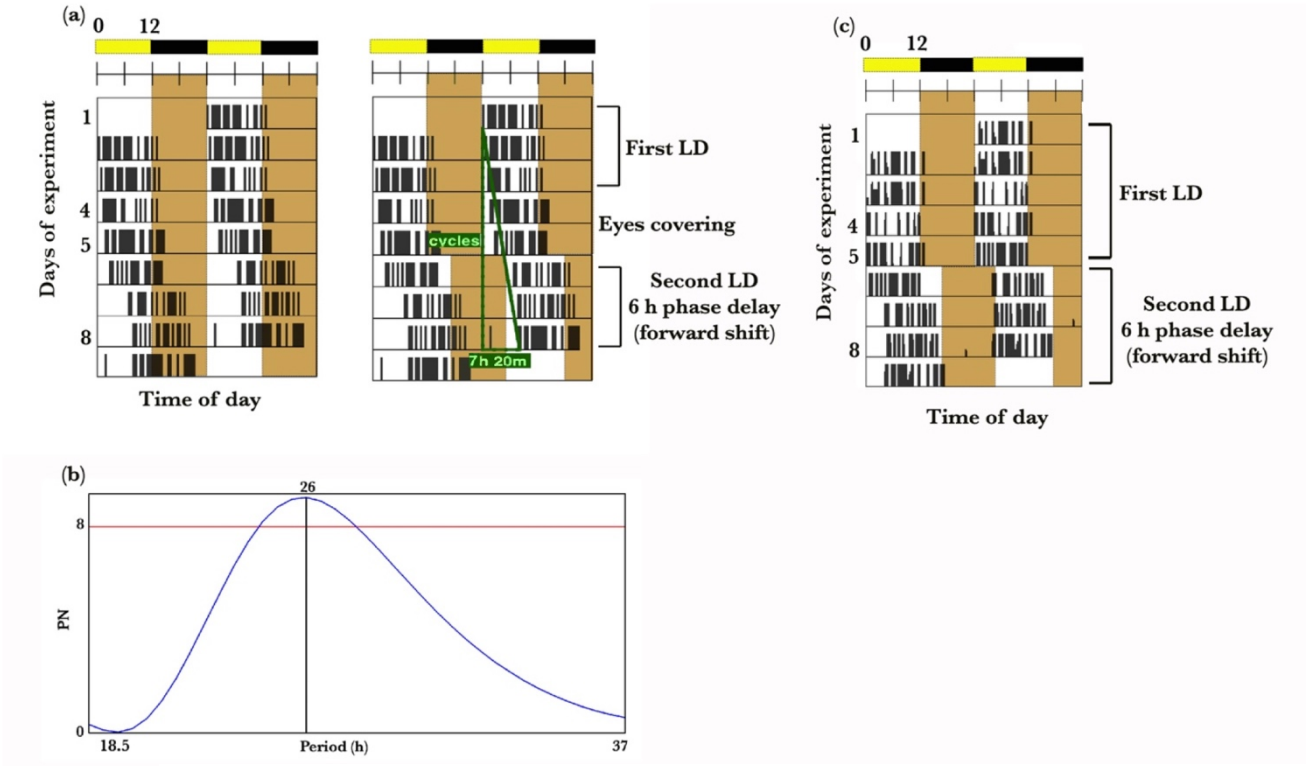

Fig. 3. The role of anterior median eyes (AM eyes) in entrainment of *M. marina* (Salticidae). **a.** Actogram of activity under the first light/dark cycles (LD) with all the eyes uncovered then covering the eyes except AM eyes in day four, one more day in LD with just AM eyes uncovered and then a 6 hours phase delay from the day six. The coloured strips (brown) indicate dark phase (scotophase). The right actogram showed the usage of green eye fitted lines (a tool in ActogramJ software) for measuring the phase angle of onset of activity towards any light-on time in given LD cycle. Here, it shows in a typical (most alike to 60-70% of population) spider, 3 days after the 6 h delay shift, the onset of activity(without considering the 2 days of transient phase) the phase angle deferens is about 1 h and 20 min toward the light-on in new LD or 7 h and 20 min toward the last LD. In left actogram the brown strips show the change in LD cycle after the shift. The dark bars represent the active time and the white areas belong to rest time. The light on time for the start of the experiment calibrated to 12:00 in software. **b.** The Lomb-Scargle periodogram of free-running period, during three days of new LD after shift for a typical spider which shows the significant rhythm of free-running. PN is the level of significance in Lomb-Scargle analysis the rhythm **c.** Actogram of the activity under first and second LD (with 6 hours phase delay) for a control spider. In all the actograms, two days of transient phase have been eliminated from the start of each LD.

On the third day after LD change, the mean phase angles of onset of activity in spiders with just AM eyes uncovered was of 1.9 h ± 0.73, $N = 11$, $n = 9$ towards lights-on time in new LD. The control spiders, with no eyes covered, in first day of new LD showed 0.15 h ± 0.7, $N = 11$, $n = 9$ phase difference in onset of their activity towards the light on time in their new LD. Activity levels of spiders with covered AM eyes and control spiders for five days showed no significant difference ($t_9 = 0.526$, $P = 0.712$, $N = 11$, $n = 9$; Fig. 4).

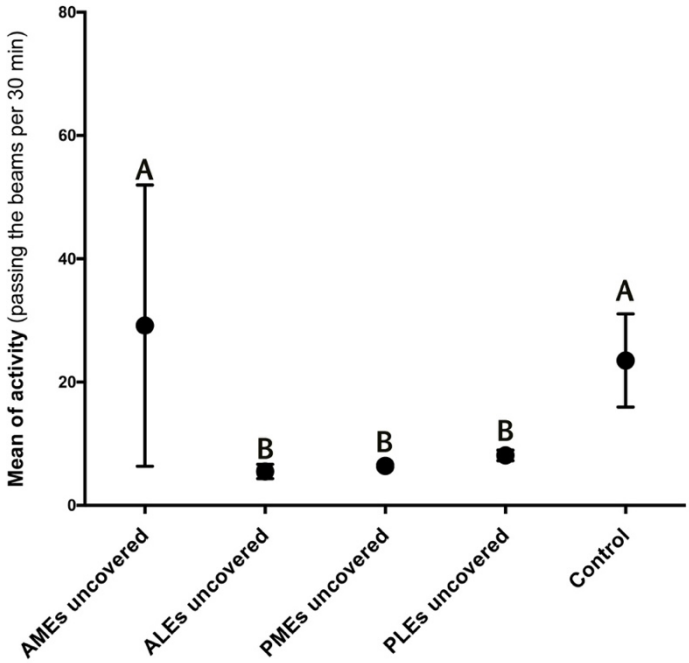

Fig. 4. Mean activity (+/- SEM) of *M. marinna* (Salticidae) spiders over 5 days of each treatment with all the eyes covered except AM eyes, AL eyes, PM eyes and PL eyes, respectively, and controls (all eyes uncovered) over the four-days (from eye covering to the end of the new LD with 6 hours phase delay). Different letters depict significant pairwise differences.

When covering the AM eyes and two pairs of secondary eyes, I got similar results regardless of which secondary eyes were uncovered. They continued rhythmic activity (days four and five) (AL eyes, Fig. 5a; PM eyes, Fig. 5b; PL eyes, Fig. 5c) and after phase delay in day six, the spiders showed an entrainment to the new LD and kept entrained until the end of the experiment. Activity levels (the mean number of spiders passes in 30 min) of spiders with covered eyes and control spiders for five days showed that activity significantly decreased (AL eyes uncovered: $t_9 = 11.08$, $P < 0.0001$, $N =$

11, $n = 9$; PM eyes uncovered: $t_9 = 8.03$, $P < 0.0001$, $N = 11$, $n = 9$; PL eyes uncovered: $t_9 = 13.53$, $P < 0.0001$, $N = 11$, $n = 9$). The results (Fig. 4) showed that activity level, when just AM eyes were uncovered was not significantly different to the control group, but the activity levels over 5 days for spiders with covered secondary eyes decreased significantly in comparison to control spiders ($F_{4,16} = 4.63$, $P = 0.011$, $N = 11$).

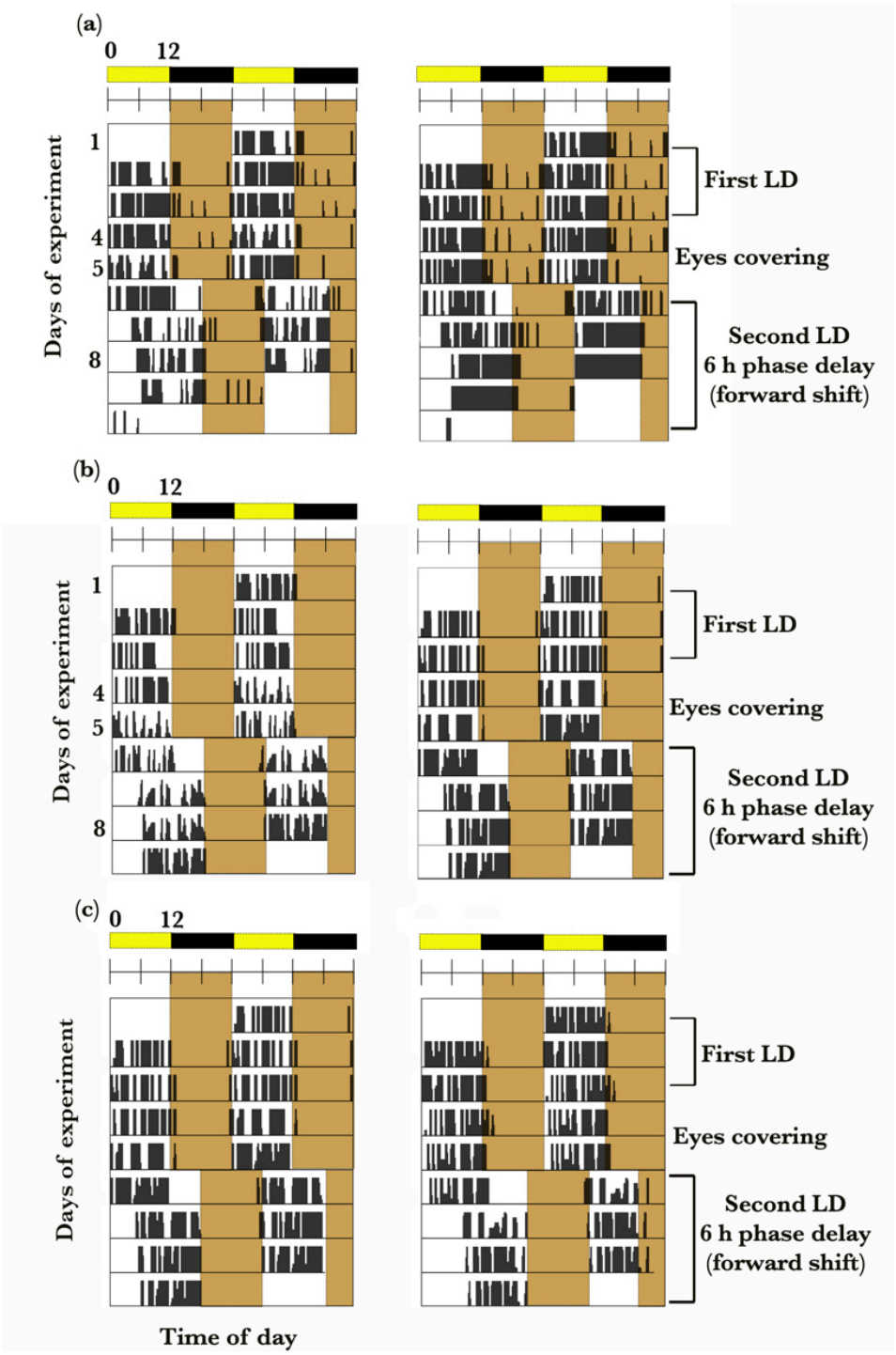

Fig. 5. The role of secondary eyes in entrainment of *M. marina* (Salticidae). Representative actograms in typical spiders with only one secondary eye available (right) and control (all the eyes uncovered) spiders (left). The coloured bands (brown) depict the scotophase. The black bars show the activity and white areas belong to rest time. As the activity of spiders with anterior-median eyes (AM eyes) covered was so low, to depict the rhythm more tangible, the upper limit of the software 20 times and lower limit of that 6 times were decreased **a.** Anterior-lateral eyes (AL eyes) uncovered **b.** Posterior-median eyes (PM eyes) uncovered **c.** Posterior-lateral eyes (PL eyes) uncovered.

Discussion

My results show that the rhythm of locomotory activity in jumping spiders is both robust and circadian and is not just a response to the absence of illumination due to the covering of multiple pairs of eyes. When only the AM eyes were uncovered, the spiders showed no entrainment in first few days, suggesting that there are no circadian photoreceptor(s) in AM eyes (or if there are, they are much more weaker than photoreceptors in secondary eyes and may be entrained in long period). Additionally, the free-running period in this timeframe showed a rhythm of >24 h activity period in a day after eye covering which was like keeping the spiders under total darkness, assuming all entrainment photoreceptors were covered suggesting existence of circadian clock(s). Before getting entrained to the new cycle, spiders with secondary eyes uncovered (pair by pair) passed two days of transient phase with arrhythmicity which is another proof for circadian clock controls. Basically, endogenous clocks (i.e., circadian) need some cycles (depends on the species, light intensities and environmental factors) to entrain to new light/dark cycle (Rensing & Ruoff 2002). In exogenous clock (i.e., hourglass model of timing) controlled behaviours (Tauber & Kyriacou 2001), or when an environmental cue has a masking effect, an immediate entrainment without need of transient phase may happen (Aschoff, J., Daan, S. & Honma, K.-I. 1982). Such that, entrainment after few cycles can be counted as evidence of circadian clock function.

Unlike AM eyes, my results showed all the secondary eyes participated in entrainment after passing the transient phase. My results corroborate previous work demonstrating behavioural specialization of jumping spider eyes (e.g., Forster 1979; Harland & Jackson 2004, Zurek et al. 2010), whereby the AM eyes are used for high acuity vision and the secondary eyes (AL eyes, PL eyes, and PM eyes), in addition to acting as motion detectors (Land 1971, Zurek et al. 2010, Zurek & Nelson 2012a, b), function in entrainment. Additionally, the loss of vision in the AM eyes led to a significant drop in activity levels.

In the lycosid, *Lycosa tarentula,* Ortega-Escobar (2002) found similar results, showing that photoreceptors in all the secondary eyes (AL eyes, PM eyes and PL eyes) were capable of behavioural entrainment, while those in the AM eyes were not. These results differ to those of insects with visual systems comprised of compound eyes and

ocelli. For instance, in *Drosophila melanogaster,* all the various photoreceptors and photopigments contributed to entrainment although they differed in their capacity to entrainment under short and long days (Rieger et al. 2003). Conversely, the photoreceptors of entrainment in the scorpion *Androctonus australis* L. (Arachnida: Scorpiones) are in the median eyes and are able to entrained by very low light intensities (Fleissner 1977).

To determine the role of eyes in circadian photoreception, in some insects, scientists have used nerve cutting (cockroach: Nishiitsutsuji-Uwo & Pittendrigh 1968; Roberts 1965; cricket: Tomioka & Yukizane 1997; and tree weta: Waddell et al. 1990). Apart from the ethical issues of sacrificing animals, Kovoor et al. (1992) showed in spiders, the AM eyes and AL eyes nerves are in a ventral position with respect to the PM eyes, so it is difficult to approach and cut them without damaging the PM eyes. The technique of eye painting has been used in crickets (Nowosielski & Patton 1963) as well as in lycosid spiders (Ortega-Escobar 2002) and showed the same results as nerve cutting. My results offered further support that interventionist approaches are not necessary to determine ocular pathways for entrainment in jumping spiders.

In many invertebrates, there is evidence for extraocular photoreceptors (which located in none-visual organs like coxa and antenna), including orb weaving spiders (Yamashita & Tateda 1983), crayfish (Page & Larimer 1976), scorpions (Zwicky 1968, 1970; Rao 1973), beetles (Fleissner et al. 1993), and horseshoe crab (Battelle 2016). Nevertheless, the existence of the extraocular photoreceptors which can entrain the ocular oscillator have been reported in other animals, such as sea slugs (Block et al. 1974). Unlike in invertebrates, the circadian photoreceptors of mammals appear to be located exclusively in the eyes (Rollag & Provencio 2003; Van Gelder & Buhr 2016); extraocular circadian photoreceptors are found in other none-mammalian vertebrates (see Underwood & Groos 1982; Foster et al. 1994). I suspected that the secondary eyes would be responsible, as Ortega-Escobar (2002) showed there are no extraocular photoreceptors in the prosoma of the lycosid spider *L. tarentuala*. And my results in this regard concurred with those of Ortega-Escobar (2002).

I suggest investigation of the role of the polarized and non-polarized light, as well as the different light intensities, in salticid ocular circadian entrainment. Kovoor et al. (1993) showed that, in ventral photoreceptors of AM eyes, successive lines of rhabdoms are oriented orthogonally to each other, suggesting detection of polarized

light; this kind of arrangement cannot be seen in the secondary eyes. Eakin & Brandengerger (1971) made a similar, but uncorroborated, suggestion for the AM eyes of salticids (see Ortega-Escobar, 2017 for a review on polarized-light vision in spiders). The similarity in the ultrastructure of lycosid and salticid eyes, makes this parallel especially interesting. In lycosid spiders, Kovoor et al. (1992, 1993) showed a different central projection of the AM eyes and of AL eyes to PL eyes, and PM eyes. The absence of a projection from AM eyes to neuroendocrine cells may explain the absence of entrainment to LD when just these eyes are uncovered (Ortega-Escobar 2002). Additionally, Kovoor et al. (1999) showed that in the rhabdoms of the AL eyes, PL eyes, and PM eyes there is a clear circadian rhythmicity, which is less clear in the rhabdoms of the AM eyes. Pursuing this line of research in salticids should be rewarding.

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Chapter 8

Discussion

Summary of main findings

In this thesis, I investigated different aspects of light-related temporal behaviours in jumping spiders (Salticidae) using the Locomotory Activity Machine (LAM) as my primary methodology. The LAM was originally designed for use with *Drosophila,* and posed some specific challenges with spiders. For example, in *Drosophila,* gel and agar can be placed at the end of the glass tubes to feed the insects during the experiment, but spiders need live prey, whose movement naturally would interfere with counting by the infrared lights in the system. Consequently, I had to remove the spiders every 3-4 days to feed them and was unable to perform the long-term experiments which provide more accurate data, especially for free-running period measurements (Seyfarth 1980). I modified the system by adding the mesh on end of the tube and a micro water cylinder at the other end. However, due to their highly visual nature, salticids could potentially recognise conspecifics and display courtship or aggressive responses to each other between nearby tubes. To minimise this, I separated males, females and juveniles during experiments, but this would not prevent potential aggressive displays between spiders. Possibly the best way to address this issue is to test very few spiders at a time, vastly slowing down data collection. Nevertheless, I was able to perform several shorter-term experiments using my modified methods and explored temporal behaviour at various levels, starting with the investigation of circadian patterns of locomotor activity, continuing with the effects of light properties on activity patterns and finally ending with an investigation of which eyes were responsible for entrainment.

I began by testing salticid responses to photoperiodism in order to investigate the effect of day light hours and temperature, and their interaction, on locomotor activity in four species (*Marpissa marina*, *Trite planiceps, Servaea incana* and *Portia fimbriata*) of jumping spiders. Circadian activity patterns were different between species and, within species, also differed depending on temperature and photoperiod. For example, *Portia* (a tropical species) was the most active species, while the temperate New Zealand species *Marpissa* and *Trite* had similar overall activity levels. Additionally, there were relatively consistent sex/age class differences, with females typically being the most active group. Generally, salticids started their day earlier and were more active in summer photoperiods and later in winter photoperiods. All species

except *Portia* tended to ramp up activity later in the day during the short photoperiod compared with the long photoperiod; in *Portia* this only was seen at the colder temperature, and irrespective of the photoperiod, suggesting that this species was more sensitive to thermal changes than the temperate species. Overall, these results suggest that the temperate species studied here were more sensitive to photoperiod than temperature and the tropical species were more sensitive to temperature.

I then investigated the effects of thirst, hunger, food quality, and mating status (virgin, mated, after egg laying) on the locomotor activity of female *Marpissa*, a generalist predator, and *Portia*, a specialist predator of other spiders (araneophagy) (Jackson 1992). Despite the influence of non-photic drives on locomotor activity, the diel pattern of activity is retained irrespective of physiological or reproductive drives. In both *Marpissa* and *Portia*, there was no difference between hungry spiders and those fed on flies, yet *Portia* fed on their preferred prey (spiders) were significantly more active, suggesting that for this specialist predator, spiders provide nutrition that allows spiders to be more active. In both *Marpissa* and *Portia*, thirsty spiders were more active than spiders that had water available to them. Reproductive status strongly affected activity levels, with the most notable, if perhaps not surprising, finding being that spiders that had recently laid eggs became almost inactive. However, it is interesting that even in this group with such low activity levels, the patterns of activity throughout the day were similar to the other groups.

In the first almost long-term experiment on spiders in which a series of light-regime phase shifts was performed, I found *M. marina* are very resilient to phase shifts up to 10 h in a forward direction (delay phase). Cycles after the advance shifts (shorter days) showed the same size of latency in the onset of activity, which was the same as advancing the onset of activity in the free-running period which I took to represent the actual circadian clocks. Under constant lighting conditions, latency to onset of activity was about 1.5 h advanced with respect to initial entrainment (i.e., 7.5 h different to entrainment). In contrast, if there was a phase delay, latency was halved and activity levels were significantly higher (i.e., 3.5 h difference to entrainment) compared with phase advances, in practice resulting in an anticipation of lights-on of about 2.5 h with respect to basic entrainment. The phase delay effectively shortened night length (or lengthened the photoperiod of the first day), by 6 h. In this case, I found *M. marina* became active much sooner with respect to their last entrainment regime. In this study,

on first day of several of the advance phase shifts, I reduced the day length and expected a decrease in the free-running period after a 6 h advance shift with respect to the previous free-running period, but found no such aftereffect. It has been suggested that animals at high latitudes experiencing long photoperiods with very short summers will have damped circadian rhythmicity, and must respond quick to photoperiod for survival (Wegis et al. 1997; Merilä et al. 2000; Angilletta 2001; Bradshaw et al. 2003; Laugen et al. 2003; Uller & Olsson 2003; Lindgren & Laurila 2005). These animals may rely on hourglass mechanisms. In *M. marina*, I found significantly shorter latency (faster response) to the onset of activity after a phase delay, but in no case found an immediate phase-shift in keeping with the new light regime, as would be expected in hourglass models. In fact, it was notable that irrespective of experiencing 6 or 10 h advance shifts, spiders maintained a remarkably close to 7.5 h delay with respect to their main entrainment, meaning that their activity would have begun roughly 90 min after lights on. These results provide no evidence that *M. marina* keep hourglass model of timekeeping, and instead rely on circadian clock models with a period of close to 24 h.

In Chapter 5, my results suggest that the ability of *M. marina* to anticipate the start and end of daylight hours is not related to light intensity during the scotophase (dark cycle), but to illumination during daylight hours. At normal light intensity (diurnal illumination) accompanied by a total darkness cycle or a moonlight cycle (nocturnal illumination), *M. marina* can rely in their circadian clock to anticipate the start and end of the day by one to two hours, showing clear diurnal templates. Under constant light, activity levels were similar for all treatments containing some light (including moonlight), although under constant intense light, the locomotor activity of spiders showed some arrhythmicity, with a masking effect being found, in which *M. marina* were no longer able to anticipate lights-on and lights-off. Depending on light conditions, the direction of free-running periods differed: under constant normal light, the period reduced to < 24 h, while under moonlight intensities, the period increased to > 24 h. These finding led me to further investigate this area, and, in Chapter 6, I showed depending on the intensity of light, constant light is able to disrupt circadian rhythmicity in *M. marina* , with a variety of responses like reduced locomotion and an increase in tau to complete arrhythmicity (Pittendrigh 1976, Aschoff 1981). I found intense constant light induces hyperactivity, which disrupts rhythmicity, with only some animals exhibiting a weak rhythm, and only during the first four days. I also

investigated the trends of non-significant rhythms, finding that the salticid oscillation system 'tries' to keep its rhythm in first days of exposure to bright light, where the free-running period tends to be > 24 h. While spiders became arrhythmic under constant intense light, they showed strong, > 24 h, rhythmicity in constant dim light. This activity pattern was opposite to that in constant darkness. My results suggest total darkness may act as a masking factor on the spider's clock, such that they lost their rhythm for more than a week, but returned to a shortened period of rhythmic activity after 10 days. Under constant dim light, there was a delay in the onset of activity compared with constant intense light, further supporting the idea that under dim light, the length of the free-running period in *M. marina* tended to be > 24 h. In addition, a lengthening of the free-running period was seen under the very first days of intense constant light such that, in contrast to intense darkness, irrespective of light intensity, under constant light conditions, the free-running period was lengthened. Altogether, the results from these two chapters suggest that the circadian oscillator(s) of *M. marina* has (have) a powerful organization which is strongly influenced by light intensity.

Finally, I sequentially covered the four pairs of salticid eyes to determine which pair/s were capable of entrainment. I found clear evidence that the antero-median, or primary eyes couldn't entrain *M. marina* to a changed cycle, suggesting that there are no circadian photoreceptors in this pair of eyes. In contrast, every pair of secondary eyes (the anterior lateral, posterior lateral and posterior median eyes) was capable of entrainment. This finding is in accordance with the only other study which has been done to investigate the role of spider`s eyes in entrainment, which was done in the family Lycosidae, a primarily nocturnal family of spiders (Ortega-Escobar 2002).

Overall, salticids have a circadian clock which is strongly diurnal and is affected by factors other than light, including temperature, mating status and feeding status. However, even at very low levels, light is the primary factor affecting the diel patterns of activity of salticids, which is mediated by photoreceptors in all pairs of secondary eyes, but not by the primary eyes.

Future directions

Despite considerable evidence of species-specific variation in salticid behaviour and life histories, salticids are a closely-related group (Jackson 1978; Cutler 1982; Jackson 1985; Logunov 1997; Punzo 2000; Bartos 2005; Ceccarelli & Crozier 2007; Jackson et al. 2008; Pekar & Jarab 2011), with differences largely considered to be 'exceptions to the rule'. This is surprising, given that the Salticidae have over 5800 (and counting) described species in over 580 genera and are the largest and most diverse spider family (Maddison 2015, Platnick 2015), and despite being poikilothermic, are found in a wide variety of habitat types encompassing all non-polar terrestrial ecosystems (Maddison 2015). Besides the variation in their habitat types, they exhibit a huge variety of lifestyles. For example, while *Marpissa, Servaea* and *Trite* hunt insects, like most salticids (Jackson & Tarsitano 1993), *Portia* is a highly unusual salticid which has a specialized predatory behaviour for hunting spiders (Jackson & Hallas 1986; Nelson & Jackson 2011a, b). My finding that *Portia* was the most active species, especially when fed on spiders rather than flies, suggests that the need for specific nutrients derived from a specialized prey for development (Li & Jackson 1997) might lead to higher levels of activity. This hypothesis could be tested using the salticid *Evarcha culicivora,* which specialises at hunting female *Anopheles* mosquitoes that have fed on blood (Jackson et al. 2005; Nelson et al. 2005) and also requires these prey for development (Deng Chan 2016). Future studies looking at the relationship between circadian patterns, activity levels, physiology and predatory behaviour in multiple species should be of interest. For example, while I found a masking effect for 300 lux light on the circadian clock of *Marpissa*, which lives in a very bright environment, the intensity of light which causes masking effects may differ dramatically in other species with different visual sensitivity, and ambient light in their habitat, such as *Portia*, whose habitat is dimly lit rainforest.

Interestingly, while the individual molecular components of the circadian timing mechanism are not homologous in all cases, the molecular mechanism which produce rhythmicity are very similar between animal groups (Dunlap 1999), such that basic information about the clock functions, which has been well-studied in *Drosophila* (Zhang & Emery 2012), likely also works in spiders. Essentially, the circadian

transcription factors CLOCK (CLK) and CYCLE (CYC) constitute a heterodimeric complex which enhance the transcription factors (*period* (*per*) and *timeless* (*tim*) (Tataroglu & Emery 2014). Additionally, *per* and *tim* condense and phosphorylate during the day and night according to the normal function of animal and make CLK and CYC active and inactive during the rest/active cycles. Furthermore, a cryptochrome (CRY), which is a blue-light photoreceptor, can get the ambient light and bind to *tim* to affect and restart the cycle, which is why light is a zeitgeber that resets the circadian clock. If the clock in animal or even in a tissue is assumed Cry-dependent, with a mutation in CRY photoreceptors with a defect function, we can't expect entrainment to a new L:D cycle (after a shift). Further molecular research in CRY-dependent pathways in spiders, if they have these, would be of comparative interest.

Circadian rhythms are controlled by the circadian clock located in the central nervous system (optic lobe or central brain) (Helfrich-Forster et al. 1998; Tomioka & Abdelsalam 2004). In addition to the central clock, there are clocks in different peripheral tissues (e.g., compound eyes, antennae). While the role of the central clock may be the global control of rhythms in an organism, peripheral clocks create a specific temporal structure for the unique functions of those tissues. Molecular studies on fruit flies, butterflies, moths and crickets (Hege et al. 1997; Plautz et al. 1997; Merlin et al. 2006; 2009; Uryu & Tomioka 2010) have illustrated that peripheral tissues show rhythmic expression of clock genes that underlie the temporal 'behaviour' of the tissue, although why many tissues have temporal expressions in their physiology is often unclear. However, further studies across species would be useful to determine the roles of the peripheral clocks in animals, including in spiders, which my work did not address. An interesting avenue for further research might be a comparative investigation on the photoreceptors in the eyes of several species within the Class Arachnida. This study could inform us about whether the secondary eyes are controlled independently or by a central clock and whether the antero-median eyes are controlled by peripheral clocks, and could answer the question of why the photoreceptors related to entrainment are located in secondary eyes.

Generally, in central clock (s) there are two separate pathways for photic entrainment in invertebrates (as studied in *Drosophila*). One is the CRY pathway (Koh et al. 2006; Peschel et al. 2009) which is a molecular avenue and the other is through the external photoreceptors, such as compound eyes and ocelli (Helfrich-Forster et al.

2001) which counts as neural one. Since peripheral clocks have no direct neural connection from the external photoreceptors, their entrainment mechanism may differ from that of the central clock, although they may be mediated indirectly by unknown pathways through external photoreceptors. The molecular mechanisms and functions of the circadian clock (s) differ on a tissue-dependent and species-dependent basis. Given the differences in behavioural entrainment of the primary and secondary eyes of salticids, it would be useful to investigate the CRY pathways in the antero-median eyes and central clocks in the lateral eyes. Generally, in photic entrainment subject, diversification between CRY-dependent (molecular) and external photoreceptor-dependent (neural) pathways can be an interesting issue. Additionally, the sensitivity of visual systems can change throughout a 24 h period. For instance, the compound eyes of crickets and cockroaches show a clock-controlled rhythm with a sensitivity peak during the night, which is controlled by a clock within the optic lobe (Tomioka & Chiba1982; Angier-Wills et al. 1986), while the responsiveness of interneurons is under the control of the circadian clock (Kaiser & Steiner-Kaiser 1983; Tomioka et al. 1993). If we assume that the rhythmic behaviour of jumping spiders to the photoperiod is related to rhythmic eye sensitivity over the day, it would be interesting to investigate the molecular sensitivity of jumping spider's eyes and their related peaks. This could be done through finding the related enzyme, making it/them active or inactive, and expose spiders to the desired light intensity to determine the photolyase mechanism/s, as damaged enzymes can be evidence of light sensitivity (Albrecht et al. 2007).

Recent comparative studies on the clock mechanism using molecular and genetic techniques have revealed the diversity and complexity of peripheral clock systems in insects (Tomioka & Matsumoto 2010). Thus, it seems very promising to investigate the clock systems comparatively across spider species that have different life-styles, circadian patterns of activity, and physiological requirements (e.g., sit-and-wait predators versus active specialised predators of specific prey). Future studies may reveal how the peripheral clocks have been altered or conserved through selection at several levels of organisation.

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Appendix 1

Video S1: A short video for the general methods of locomotor activity measurement which used in all experiments. Link: click here

Appendix 2

Activity measurements supplementary information

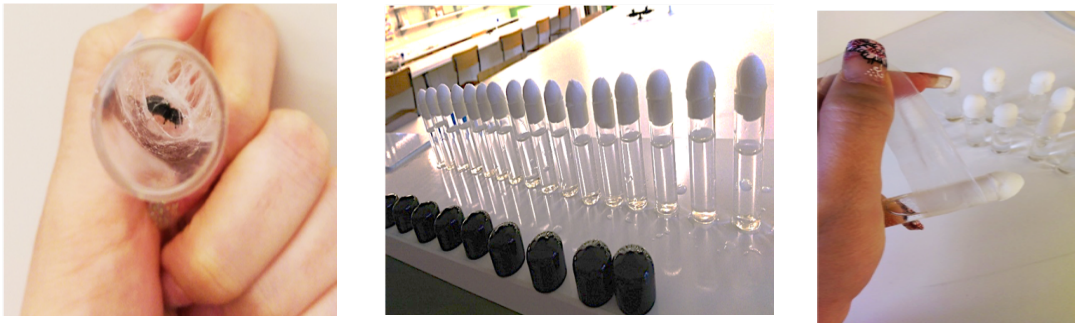

Fig S1. Preparation of the micro cylinders for supplying the water over the experiments. The test tubes which covered by silk, replaced after each experiment.

Fig S2. Activity measurement by means of (LAM) (LAM25, TriKinetics, Waltham, MA, USA) monitors in incubator which makes it possible to control the light intensity, day light hors and darkness as well as temperature.

Appendix 3

Chapter 2: The effect of photoperiod and temperature on the locomotory activity of four species of jumping spiders

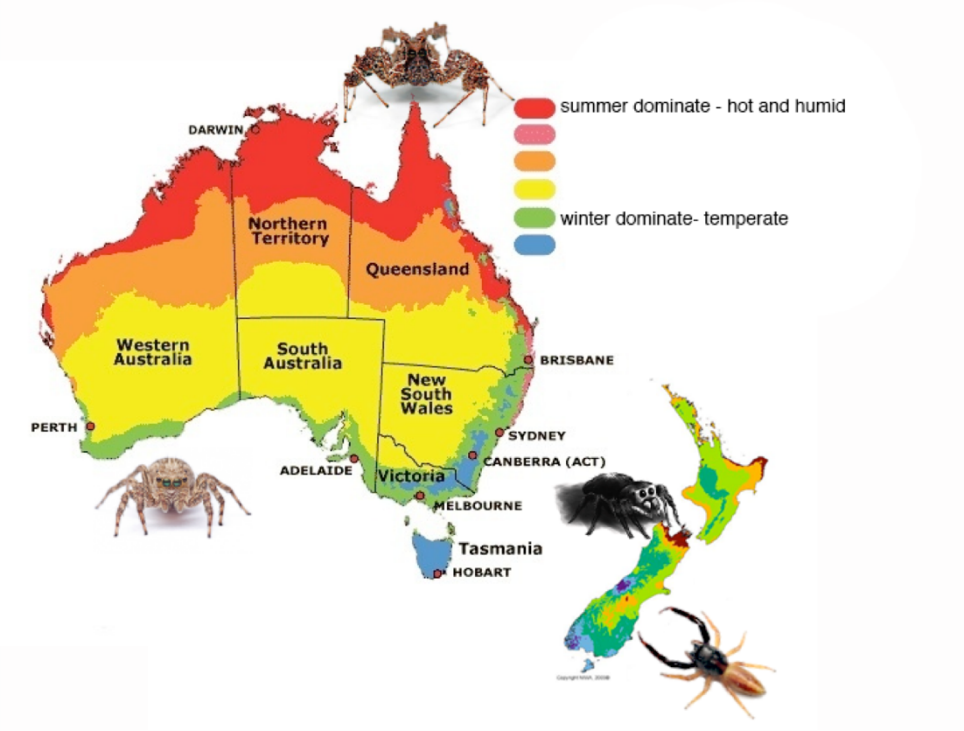

Fig. S3. The origin of the species of jumping spiders (*Marpissa, Trite, Portia, Servaea*) which used in experiment.

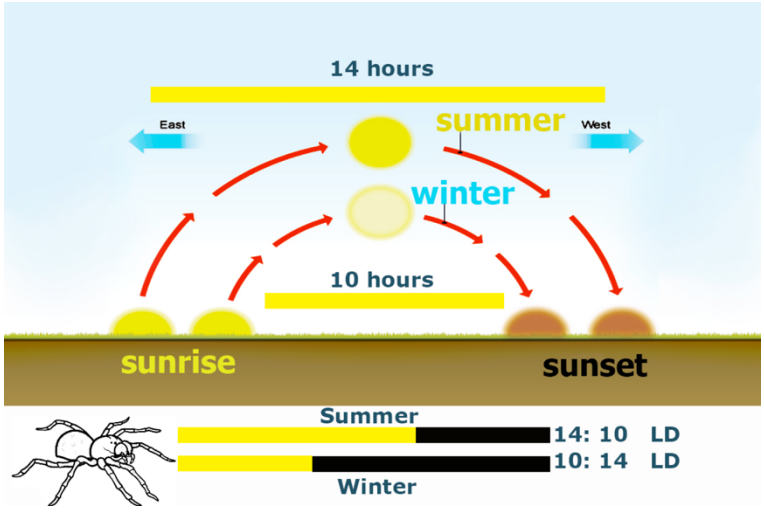

Fig. S4. Diagram of photoperiod design which stimulates winter and summer day light hours in the incubator.